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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY
BY
C. R. HARINGTON

ASSOCIATE EDITORS
S. J. COWELL
F. DICKENS
and
F. J. W. ROUGHTON

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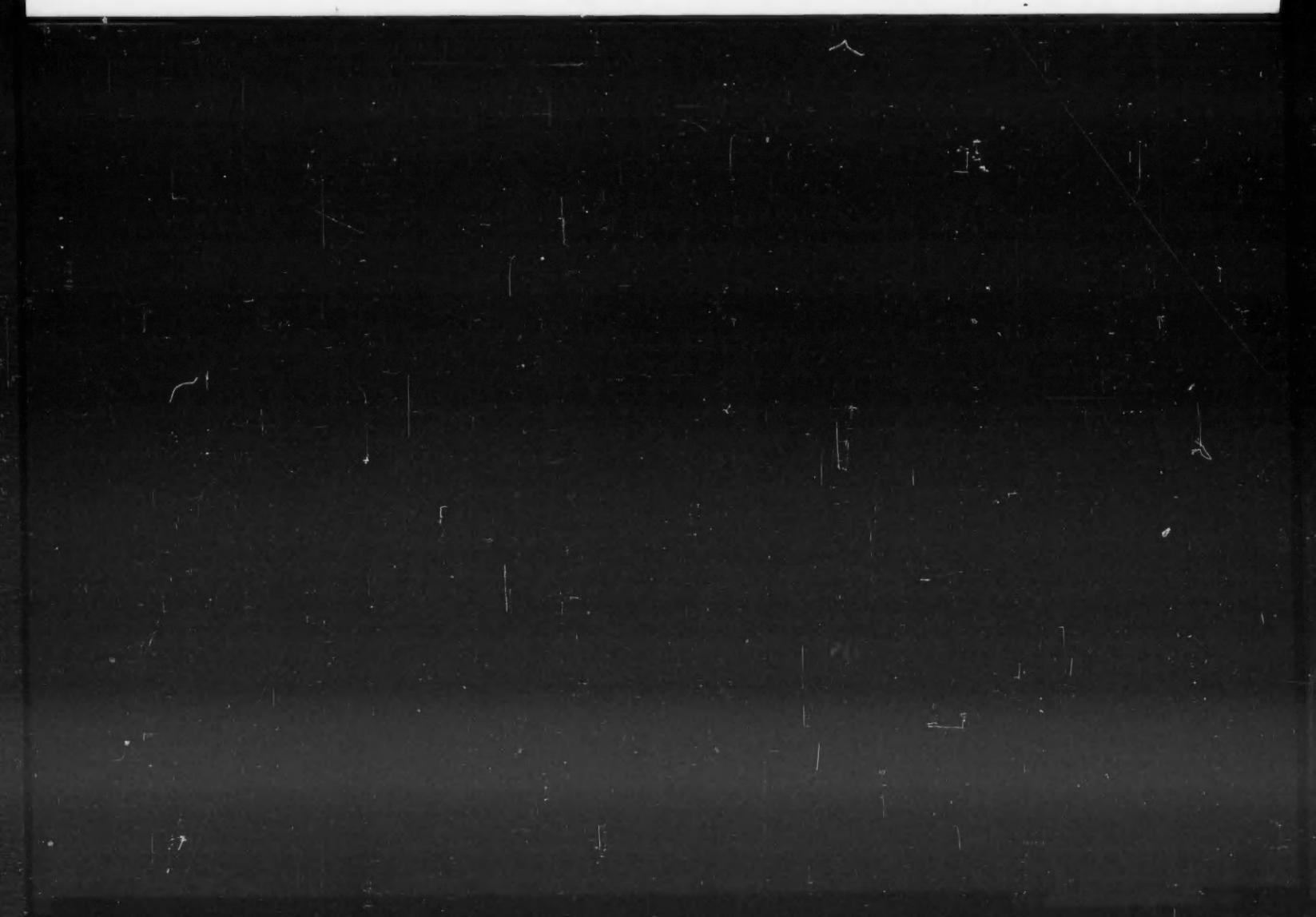

1. CHANGES IN PHOSPHATE DISTRIBUTION DURING ANAEROBIC GLYCOLYSIS IN BRAIN SLICES

By MARJORIE GIFFEN MACFARLANE and HANS WEIL-MALHERBE

From the Biochemical Department, Lister Institute, London

(*Received 9 November 1940*)

It has been shown that the changes in phosphate distribution during fermentation in the living yeast cell are so consistent with the theory, developed from experiments with cell-free extracts, that anaerobic glycolysis takes place by intermediate phosphorylation that there can be little doubt that the processes are analogous [Macfarlane, 1939]. It was pointed out at the same time that in cells the normal glycolysis may be maintained by a cycle of P transference which involves such small amounts of P that the isolation or detection of phosphorylated intermediate products may be extremely difficult. There has been considerable argument over the existence in certain tissues, e.g. brain, tumour, retina, embryo etc., of a non-phosphorylating type of glycolysis, though the preparation of cell-free phosphorylating extracts from these tissues [Geiger, 1940; Boyland & Boyland, 1935; Kerly & Bourne, 1940; Meyerhof & Perdigon, 1939] supports the view that an analogous phosphorylation takes place in the cells. The fact that glucose can act as a phosphate acceptor in dialysed brain dispersions [Ochoa, 1940] is even more convincing evidence of the correctness of this theory. We have examined the changes in P distribution which take place during anaerobic glycolysis and autolysis in slices of rabbit brain and find that they are consistent with the view that the glycolysis is dependent on phosphorylation.

Methods. The rabbits were killed by a blow on the neck and the brain removed immediately into Ringer solution. Slices were cut in the manner usual for manometric experiments and kept aerobically in Ringer solution at room temperature until sufficient material was obtained. Roughly equal amounts of tissue were drained and placed in tared flasks containing the medium (0·5 g. to 5 ml. medium) and the wet weight of tissue obtained by reweighing. The flasks were immersed in a thermostat at 37° and shaken mechanically. A stream of N_2, containing 5 % CO_2, was passed through for 3 min.; the outlet was then closed and the flasks were left connected to the gas cylinder. Enzyme action was stopped by the addition of trichloroacetic acid in final concentration 3–5 %. Approximately 20–25 min. elapsed between the death of the animal and the start of anaerobic incubation, at which point the zero value was taken. It was anticipated that this method of weighing, though it causes the minimum injury to the slices, would be subject to considerable error owing to occlusion of liquid in bunches of slices; it was found, however, that though there were occasional anomalous values the results of manifold experiments were on the whole in good agreement. The average dry weight was 12·5 % of the wet weight.

The medium used was a phosphate-free modification of Krebs & Henseleit [1932] Ringer solution, saturated with the N_2/CO_2 mixture, the K_2HPO_4 being replaced by an amount of KCl sufficient to give the same ionic concentration of K; for glycolysis it contained in addition 0·2 % glucose. An experiment was

performed in which the medium used was horse serum which had been dialysed against P-free and bicarbonate-free Ringer solution. Before use the serum was inactivated by heating for 2 hr. at 56°; bicarbonate was then added in the required amount and the serum saturated with the gas mixture. Contrary to expectation the serum did not decrease the autolysis of the tissue, as manifested by the liberation of phosphate, and its use therefore offered no advantage over Ringer solution. Estimations were made on aliquots of the trichloroacetic acid filtrate as follows: total P after ashing, and inorganic plus creatine P by direct estimation, by Briggs's method; pyrophosphate by calculation from the hydrolysis curve in *N* HCl at 100°, pyro-P=Δ (7 min.–0 min.)—Δ (30 min.–7 min.); hexosediphosphate-P by the amount of alkali-labile P formed by incubation with zymohexase (dialysed muscle extract) in presence of 0·1 *N* KCN at *p*H 6·8 and 37° for 15 min.; lactic acid by oxidation with ceric sulphate [Gordon & Quastel, 1939] and iodimetric titration of the acetaldehyde-bisulphite compound. Values are expressed in mg./g. dry weight.

Changes in inorganic and pyrophosphate-P during anaerobic glycolysis. Table 1 shows that the inorganic P content of slices incubated anaerobically with glucose for 30 min. was much less than that of slices incubated without glucose, both absolutely and also relatively to the total acid-soluble P, which increased noticeably during autolysis. There was, however, no absolute decrease in inorganic P compared with the initial value and therefore no direct proof of

Table 1. *Anaerobic incubation of brain slices at 37°*

The values obtained for the three experimental conditions, A, B, C, show the extent of variation in aliquots of the same brain or in similar aliquots from different brains. The horizontal line of figures is not necessarily from the same animal in A, B and C.

A Initial				B 30 min. glycolysis				C 30 min. autolysis			
	Inorganic P				Inorganic P				Inorganic P		
Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.
2·80	1·52	**54**	3·52	3·12	1·56	**50**	37·2	3·36	2·24	**67**	2·6
2·96	1·60	**54**	3·76	2·96	1·54	**52**	32·5	2·80	1·84	**66**	2·9
2·72	1·60	**59**	—	2·56	1·48	**58**	33·1	3·20	2·24	**70**	—
2·72	1·52	**56**	3·20	2·56	1·36	**53**	37·2	2·96	2·16	**73**	2·7
2·32	1·28	**55**	—	2·88	1·68	**58**	38·8	3·36	2·16	**74**	—
Average:											
2·7	1·5	**55·5**	3·5	2·8	1·5	**54**	36	3·1	2·1	**68**	2·7

esterification. The observed values were nevertheless quite consistent with the idea that the whole of the P necessary for the glycolytic cycle may be derived from adenylpyrophosphate, and that in the absence of glucose the labile P is broken down with liberation of inorganic phosphate; the fact that after short anaerobiosis brain slices lose their capacity to break down glucose [Dickens & Greville, 1933] affords some support for this view. It was thought possible that re-esterification of inorganic P might be demonstrated by allowing a partial breakdown of the combined P to take place before the addition of glucose. It was found, however, that in the absence of glucose the breakdown of the pyrophosphate-P initially present was so rapid, even at 20°, that it was complete in 3 min. (Table 2), little more than the time considered necessary to secure anaerobic conditions, and that after this time the slices had also lost the ability to form lactic acid (Table 3). In the presence of glucose (Table 2) the initial level of

Table 2. *Changes in P fractions during incubation at 37°*

Incubation time min.	Glucolysis					Autolysis		
	Total P	Inorganic P	Pyro-P	Lactic acid	$Q_L^{N_2}$	Total P	Inorganic P	Pyro-P
0	2·12	0·98	0·16	3·4	—	2·83	1·39	0·14
3	2·74	1·44	0·17	13·7	51	3·10	1·79	0
10	2·77	1·58	0·06	16·1	19	3·13	1·83	0·01
30	2·88	1·69	0·07	37·2	17	3·34	2·18	0
90	2·74	1·81	0·06	80·8	13	3·38	2·46	0·01

Table 3. *Addition of glucose after preliminary anaerobic incubation for varying times*

		37°				20°			
			Inorganic P				Inorganic P		
	Total time min.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.
No glucose	0	2·69	1·54	**57**	3·2	2·56	1·38	**54**	2·2
,,	3	2·94	1·87	**64**	3·2	2·77	1·76	**63**	2·6
,,	15	2·85	1·91	**67**	3·2	2·58	1·76	**68**	2·2
,,	30	2·99	2·18	**73**	2·4	2·74	1·90	**69**	2·8
Glucose added:									
Initially	30	2·59	1·48	**57**	32·8	2·61	1·41	**54**	16·9
After 3 min.	30	2·83	1·95	**69**	4·8	3·14	1·87	**60**	5·2
After 15 min.	30	3·02	2·10	**70**	3·0	2·88	2·13	**74**	2·6

pyrophosphate-P was maintained for a few minutes and then decreased to a fairly constant level approximately one-third of the original value. There was a concomitant decrease in the rate of lactic acid production, the $Q_L^{N_2}$ falling from a value of 50 over the first few minutes to about 15 over the next hour. The latter is that commonly reported for brain slices, but the equilibration period of 10–20 min. necessary in the manometric technique precludes the observation of a rapid initial glycolysis. The abnormally low initial value of total and inorganic phosphate in this experiment is probably due to the fact that the brain slices were immersed immediately after cutting in glucose-Ringer solution, instead of glucose-free Ringer; it was found on several occasions that this procedure prevented the liberation of some acid-insoluble compound during the aerobic storage of the slices. On the presumption that the pyrophosphate-P is derived from adenyl-pyrophosphate, the amount of pyro-P available for the formation of hexose-phosphoric esters, etc. is of the order of 0·1–0·2 mg. P per g. dry tissue, roughly only 5 % of the total acid-soluble P.

Estimation of hexosediphosphate. From previous experiments with yeast it seemed probable that the ester present in largest amount would be hexose-diphosphate and attempts were made to identify this ester using larger quantities of tissue. The experiments were made with brain slices from three or more rabbits (10–20 g. tissue), the trichloroacetic acid filtrates from the individual experiments being pooled for fractionation. The filtrate was adjusted to *p*H 8 and treated with barium acetate and then with alcohol to 10 % final concentration. The crude Ba precipitate was dissolved in HCl and the Ba removed as sulphate. In most of the experiments attempts were made to remove the inorganic P by addition of $MgCl_2$ and NH_3, but a considerable amount of organic P was carried down with the precipitate; in the third glycolysis experiment (cf. Table 4) the estimation of hexosediphosphate was made without

further purification. The accuracy of the method used—enzymic formation of alkali-labile P—was in this case seriously limited by the tenfold excess of inorganic P over organic P; the method is, however, the most specific available and the positive results quoted are outside the experimental error. There was only a poor recovery (30–70 %) of the organic P calculated to be present in the crude Ba precipitates, probably owing to adsorption on the $BaSO_4$ precipitate. Table 4 shows that in two out of the three glycolysis experiments hexosediphosphate was detected; the amounts present, though absolutely very small, were of the order expected from the amount of pyrophosphate available. No hexosediphosphate was found in the autolysed slices and only a trace in the unincubated slices.

Table 4. *Fractionation of acid-soluble P compounds in brain slices*

	mg. P/g.				
		Autolysis	Glycolysis		
	Initial	20 min.	20 min.	20 min.	15 min.
Total P	2·90	3·33	3·25	3·28	3·28
Inorganic P	1·56	2·16	1·66	1·75	1·84
Pyro-P	0·24	—	—	0·20	0·11
Hexosediphosphate-P	0·01	0	0·03	0·09	0
P in soluble Ba salts	0·80	0·93	0·89	1·02	0·88

The fractions of acid-soluble organic P hydrolysed between the 7th and 180th min., and that not hydrolysed in 180 min., remained almost the same during anaerobic incubation with or without glucose. The amount of P present in compounds with soluble Ba salts was also of the same order in all experiments. It therefore seems unlikely that these compounds are precursors of lactic acid but their nature was not investigated. According to Tschaiisow [1932] and Palladin [1934] hexosemonophosphate is present in brain tissue, but the identification of this "lactacidogen" was very unspecific. Booth [1935] has isolated from both sheep and horse brain a compound, identified as a choline ester of sphingosinephosphoric acid, which accounted for at least 50 % of the soluble organic P present in the tissue. The soluble fraction probably includes the nucleotide-P of the coenzymes.

Discussion

It was shown by Engel & Gerard [1936], working with lobster and crab nerve, that during anaerobiosis there is an extensive hydrolysis of both phosphoarginine and adenylpyrophosphate with a corresponding rise in inorganic phosphate; both these compounds were resynthesized by subsequent exposure to O_2. A rapid breakdown of phosphocreatine takes place in mammalian brain [Kerr, 1935]. We have shown in the present experiments that on anaerobic incubation of rabbit brain slices there is a very rapid breakdown of pyrophosphate-P, followed by a further steady liberation of inorganic P from a source previously insoluble; in the presence of glucose there is only a partial breakdown of pyrophosphate and a very slight increase in inorganic P. It is clear that the maintenance of the inorganic phosphate at its original level during glycolysis is not in itself proof of a phosphorylating glycolysis, since the continuous liberation of P is presumably only one aspect of a tissue autolysis which would be largely prevented by the utilization of glucose, irrespective of the mechanism of glycolysis. The correlation

however between the rapid loss of glycolytic capacity, observed first by Dickens & Greville [1933], and the rapid breakdown of pyrophosphate, and also between the aerobic restoration of this capacity [Quastel & Wheatley, 1937] and the aerobic resynthesis of adenylpyrophosphate in similar tissue [Engel & Gerard, 1936], affords considerable support for the view that the anaerobic glycolysis in brain tissue takes place through the intermediate formation of phosphoric esters by transference of P from adenylpyrophosphate. This view is strengthened by the detection of hexosediphosphate in the glycolysing tissue. The amount of P involved in the glycolytic cycle in brain tissue with a $Q_L^{N_2}$ about 15 is apparently of the order 0·1–0·2 mg. P per g. dry tissue; in living yeast the corresponding figures are of the order 1–2 mg. P per g. and $Q_{CO_2}^{N_2}$ 100 to 200. The glycolytic rate may therefore afford a rough guide to the amount of P engaged in the cycle.

It appears from the high rate of glycolysis in the first few minutes that the values of $Q_L^{N_2}$ generally obtained in manometric experiments are considerably lower than the maximum glycolytic capacity of the tissue. This is consistent with the occasional observation of an aerobic glycolysis which exceeds the normal anaerobic glycolysis, as in certain experiments with NH_4^+ or maleic acid [Weil-Malherbe, 1938] and with guanidines [Dickens, 1939]. This fact bears on the significance of the Pasteur-Meyerhof quotient, since it is obviously difficult to correlate the aerobic and anaerobic activities of a tissue if the enzyme system is so greatly affected by anaerobiosis.

Geiger [1940] has shown that during glycolysis in brain extracts inorganic phosphate is esterified with formation of hexosephosphoric esters, while adenylpyrophosphate, cozymase and magnesium are essential coenzymes for glycolysis. His main objection to the view that hexosephosphoric esters are intermediaries in the glycolysis is the fact that the rate of lactic acid formation from these esters by brain extract is much lower than that from glucose, and is not accelerated by arsenate. The imbalance between the rates of formation and breakdown of hexosephosphoric esters in cell-free extracts clearly does not exclude phosphorylation as the main path of glycolysis; it is a phenomenon which is characteristic of alcoholic fermentation by yeast juice and one to which we owe the isolation of hexosediphosphate by Harden & Young. It has been shown that the rapid fermentation of phosphopyruvic acid, and therefore of its precursor hexosediphosphate, is dependent on the presence both of glucose (or some other acceptor) and inorganic phosphate [Meyerhof & Kiessling, 1935]. The accelerating action of arsenate is apparently an even more complex process for which a number of explanations have been offered [Needham & Pillai, 1937; Meyerhof *et al.* 1937; Negelein & Brömel, 1939]; it seems that it may be influenced in certain cases by factors not yet understood, for Pillai [1938] observed that, contrary to the usual experience, lactic acid formation from glycogen and hexosediphosphate by his fresh muscle extracts was not accelerated by arsenate, though the rate of dismutation of triosephosphate and pyruvate was increased.

Summary

During anaerobic glycolysis by rabbit brain slices the inorganic phosphate is maintained at its initial level while the content of pyrophosphate-P falls to about one-third of the original. Hexosediphosphate was present in small amounts in the glycolysing tissue. Anaerobic incubation without glucose led to a very rapid disappearance of pyrophosphate-P, with a simultaneous loss of glycolytic capacity. These results are considered to support the view that glycolysis is

maintained by a cycle of P transference involving approximately 0·1–0·2 mg. P per g. dry brain tissue.

One of us (H. W.-M.) wishes to acknowledge the hospitality of the Lister Institute during August and September.

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2. METABOLISM OF NORMAL AND TUMOUR TISSUE

19. THE METABOLISM OF INTESTINAL MUCOUS MEMBRANE

BY FRANK DICKENS AND HANS WEIL-MALHERBE

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(*Received 30 December 1940*)

STUDIES of the metabolism of intestinal mucous membrane are not numerous and seem to have been restricted to the mucosa of the large intestine [Warburg *et al.* 1924; Rosenthal & Lasnitzki, 1928; Bumm *et al.* 1934]. Dickens & Šimer [1930, 2] found the R.Q. of mucosa from the ileum to be low (0·85), but this result was not considered surprising in view of the low glycolytic activity reported by Warburg *et al.* [1924]. Weil-Malherbe [1938], however, observed with mucosa from rat jejunum a metabolism fundamentally different from that previously recorded for intestinal mucosa. All these various results are summarized in Table 1 from which it is seen that there is an extraordinarily great discrepancy between results obtained by different authors. Thus, while according to Warburg *et al.* mucous membrane belongs to the group of tissues with very low glycolysis, such as liver and kidney, Rosenthal & Lasnitzki as well as Bumm *et al.* found a high anaerobic glycolysis in their experiments with mucosa from the large intestine. Both groups of workers reported however a low aerobic glycolysis of this tissue, though occasionally high values were observed. Weil-Malherbe, on the other hand, found with mucosa from the jejunum very high values of aerobic glycolysis and a practically complete absence of the Pasteur effect. These observations seemed to us important enough to be followed up by a more detailed investigation in connexion with our previous studies on the significance of high aerobic glycolysis

Table 1. *Metabolism of intestinal mucous membrane: published data*

Average figures have been calculated, where possible, from the authors' results.

Part of intestine	Species	Medium*	No. of exp.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	R.Q.	Author
Not stated	Rat	BR	1	−12·4	1·6	3·5	—	Warburg *et al.* [1924]
Large intestine	Rat	BR	9	−14·6	4·2	15·1	—	Rosenthal & Lasnitzki [1928]
,,	,,	Serum	2	− 9·9	1·1	10·3	—	,,
,,	Rabbit	BR	11	−11·1	2·7	14·2	—	,,
,,	,,	Serum	10	−11·2	1·4	12·6	—	,,
Ileum	Rat	PO_4-R	2	− 9·4	—	—	0·85	Dickens & Šimer [1930, 2]
Large intestine	Guinea-pig	B & PO_4-R 95% O_2	18	−14·6	4·2	15·7	—	Bumm *et al.* [1934]
		(10 or 15% O_2)	(18)	(−13·2)	(9·6)			
Jejunum	Rat	BR	4	−14·6	18·3	20·4	—	Weil-Malherbe [1938]

* BR = bicarbonate-Ringer solution + glucose.
PO_4-R = phosphate-Ringer solution + glucose.
Serum = inactivated serum + glucose.

combined with low R.Q. in tumours [Dickens & Šimer, 1930, 2; 1931, 2]. Our results confirm and extend the earlier observations of Weil-Malherbe on the mucosa of jejunum. They show that this tissue differs in some respects from the mucosa of the large intestine. As in tumour, high aerobic and anaerobic glycolyses are combined with a low R.Q. Moreover, the Pasteur effect is completely absent, even if Ringer solution is replaced by serum.

Experimental

Preparation of tissue. The intestine was first rinsed out with a fine jet of water to remove the greater part of the contents. A length of about 1 in. was then cut open and spread on a glass plate with the mucous membrane facing upwards. After careful blotting with filter paper to remove adhering moisture and solid particles the mucosa was scraped off with the aid of a microscope slide. Another slide served to pin down the muscularis. With practice the mucosa can be stripped off entirely and without laceration. After weighing on a torsion balance the mucous membrane was transferred to the manometer vessel, and another piece was dried immediately for determination of the dry weight/wet weight ratio.

Methods. Measurements of respiration and of aerobic glycolysis at 37·5° were usually carried out simultaneously by Warburg's 2-vessel method in bicarbonate-Ringer [Krebs & Henseleit, 1932] or in inactivated sheep serum. A third manometer, filled with $N_2+5\%$ CO_2, served for the measurement of anaerobic glycolysis. If not stated otherwise the medium contained 0·2% glucose. Retention in serum was determined by the method of Dickens [1939]. Aerobic glycolysis in the 2-vessel method was calculated on the basis of an R.Q. of unity. Measurements of R.Q. were carried out in phosphate-Ringer by the first method of Dickens & Šimer [1930, 1] and in bicarbonate-Ringer or serum by the second method of Dickens & Šimer [1931, 1]. Lactic acid was estimated by the method of Gordon & Quastel [1939].

Infection. Bacterial contamination, noticeable by the development of a faecal smell and a steadily rising metabolism, was encountered only in a few experiments with tissue from the lower part of the ileum or from the large intestine, and even then only in the second hr. of the experiment: such experiments were of course discarded.

Thickness of the tissue. The data collected in Table 2 show that the thickness (moist wt./area) of even the whole intestinal wall of the rat is well below the limiting thickness for complete O_2-saturation, which, for a tissue with $Q_{O_2}=15$, is 0·47 mm. in pure O_2 and 0·21 mm. in 20% O_2 [Warburg, 1923]. An adequate diffusion of O_2 was therefore assured in all experiments. The thickness of the mucosa is greatest at the upper end of the intestine and diminishes steadily towards the rectum.

Disintegration of the tissue. A considerable loss of weight is observed after pieces of intestinal mucosa have been shaken in Ringer solution at 37·5° for an hour. This disintegration varies greatly from one experiment to another, even with tissue from the same animal and the same part of the intestine, probably owing to the degree of success in stripping off an unbroken piece of mucosa in its entirety. The loss of wt. is usually higher in the small volume than in the large one, but there is no regularity. Our first experiments, which were carried out before this fact was sufficiently appreciated, were calculated on the final dry wt. as is the routine in tissue metabolism work. The results showed a considerable variation and the figures obtained were sometimes unusually high. Results of

Table 2. *Thickness of the intestinal wall and its components (rat)*

		Thickness in mm.		
		Whole wall	Mucosa	Muscularis
Jejunum		0·095	0·062	0·032
		0·105	0·069	0·024
		0·093	0·061	0·024
	Mean	0·098	0·064	0·027
Ileum		0·086	0·048	0·026
		0·071	0·044	0·023
	Mean	0·078	0·046	0·025
Colon		0·115	0·046	0·059
		0·112	0·023	0·067
	Mean	0·114	0·035	0·063

Table 3. *Loss of weight after 1 hr. shaking at 37·5°*

				% Recovery $\left(\frac{\text{Final dry wt.}}{\text{Initial dry wt.}} \times 100\right)$		
				In O_2 + 5% CO_2		In N_2 + 5% CO_2
Species	Tissue	Medium*	Initial dry wt. / Initial wet wt.	v_F = 2 ml.	v_F = 8 ml.	v_F = 3 ml.
Rat	Duodenum, mucosa	BR	0·199	59·0	49·1	64·8
		BR	0·197	48·5	67·5	78·0
Rat	Jejunum, mucosa	BR	0·211	61·6	52·1	61·3
		BR	0·239	68·5	76·0	59·3
		BR	0·226	—	—	65·6
		BR	0·198	29·4	44·6	19·6
				25·8	45·6	
		BR	0·188	57·0	41·0	
				49·0	36·0	—
				45·0	51·0	
		BR	0·197	23·0	55·5	61·5
		Serum		100·0	104·5	100·0
		BR	0·195	27·0	70·0	28·0
		Serum		108	60·0(?)	85·0
Rat	Ileum, mucosa	BR	0·193	36·1	38·3	39·4
		BR	0·184	28·4	26·5	31·8
		BR	0·170	27·5	32·4	36·0
		Serum		64·7	56·8	71·6
Rat	Colon, mucosa	BR	0·260	47·5	41·0	48·2
Rat	Jejunum, muscularis	BR	0·227	82·5	68·0	45·8
Rat	Jejunum, whole wall	BR	0·228	91·8	88·5	83·5
Mouse	Jejunum, whole wall	BR	0·234	90·5	89·8	78·0
		Serum		95·4	101	94·8

* BR = Bicarbonate-Ringer solution + glucose.

greater uniformity were obtained when the calculation was based on the initial dry/wet weight ratio. This method was used in most of the experiments to be reported; only a few of the earlier experiments have been retained where the differences within one and the same experiment serve to illustrate a point. Sometimes the results have been calculated by both methods to demonstrate the effect of the loss of weight on the calculation; it will be noticed that the figures based on the final dry weight are up to 5 times higher than those based on the initial dry/wet weight ratio.

In conformity with the decreasing thickness of the mucous membrane the disintegration is greater with material from the ileum and colon than with that from the jejunum.

There is very little loss of weight when the whole intestinal wall is used. If serum is used instead of Ringer solution the disintegration of the mucous membrane is greatly reduced; indeed the mucosa of the jejunum may be recovered almost intact after a 1 hr. experiment.

Results

Examples of the metabolism of the mucous membrane from different parts of the intestine of the same animal are given in Table 4 (cf. also Table 6). The metabolism is most active in the upper and middle parts of the jejunum though respiration was occasionally found highest in the duodenum. Usually respiration is steadier than glycolysis which is very unstable and falls to the same extent aerobically and anaerobically. This fall cannot be due to a mere mechanical breaking up of the tissue as it is also observed in serum where disintegration is

Table 4. *Metabolism of different parts of the intestine in bicarbonate-Ringer*

		Results based on initial dry/wet wt.			Results based on final dry wt.		
	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
		Exp. 1. Rat, mucous membrane					
Duodenum	0– 20	– 8·8	11·1	10·4	–21·2	23·0	13·3
	20– 40	– 8·1	7·8	6·9	–17·6	16·2	8·8
	40– 60	– 6·0	4·7	4·9	–12·1	9·6	6·2
Jejunum	0– 20	–15·6	16·5	16·4	–23·1	24·0	27·8
(upper part)	20– 40	–14·8	12·3	13·3	–22·1	17·9	22·4
	40– 60	–11·9	7·0	8·9	–16·8	10·1	15·0
Ileum	0– 20	– 5·3	5·5	4·0	–17·6	19·4	12·5
(lower part)	20– 40	– 2·1	2·0	2·2	– 7·3	7·0	6·8
	40– 60	– 1·6	1·0	1·0	– 5·5	3·6	3·0
Colon	0– 20	– 3·4	5·2	4·4	– 6·4	11·0	9·0
	20– 40	– 3·2	3·5	1·9	– 5·0	3·2	4·0
	40– 60	– 1·4	2·1	0·6	– 2·5	4·3	1·1
		Exp. 2. Rat, mucous membrane					
Duodenum	0– 60	—	—	—	–50·0	24·6	38·5
	60–120	—	—	—	–28·0	7·3	12·6
Jejunum	0– 60	—	—	—	–22·8	35·1	39·0
	60–120	—	—	—	–13·8	11·1	18·5
Ileum	0– 60	—	—	—	–13·7	14·3	15·7
	60–120	—	—	—	–10·6	11·0	11·0
Colon	0– 60	—	—	—	–16·2	7·6	23·4
	60–120	—	—	—	– 7·4	4·7	—
		Exp. 3. Mouse, whole intestinal wall					
Duodenum	0– 60	—	—	—	–16·6	20·6	13·0
	60–120	—	—	—	–13·3	15·3	9·9
Jejunum	0– 60	—	—	—	–28·2	32·1	23·3
	60–120	—	—	—	–21·0	23·0	15·9
Ileum	0– 60	—	—	—	–10·7	4·5	9·3
	60–120	—	—	—	–10·8	4·4	6·3
Colon	0– 60	—	—	—	–14·1	3·9	8·1

small. With mucosa from the ileum and colon, respiration, too, is unstable and this may well be ascribed to the greater disintegration of this tissue. If based on the initial dry weight the whole metabolism of these latter tissues is very low.

Respiration and aerobic and anaerobic glycolyses have about equal Q-values, especially if the calculation is based on the initial dry weight. This is at least true for the jejunum, whereas in the cases of the ileum and the colon the results are less consistent. Sometimes the glycolysis was as high aerobically as anaerobically (Table 4, exp. 1 for ileum and colon, exp. 2 for ileum); at other times the anaerobic exceeded the aerobic glycolysis (Table 4, exp. 2 for colon, Table 6 for ileum). In some experiments it was even observed that the glycolysis was higher aerobically than anaerobically, especially in the experiments with the whole intestinal wall of both rat and mouse (Tables 4–6). This is probably an artefact due to a more rapid autolysis under anaerobic conditions [cf. Macfarlane & Weil-Malherbe, 1941].

The high aerobic glycolysis of the isolated mucous membrane is not caused by the severance from its matrix as has been suggested for retina [Warburg *et al.* 1924]. If the whole intestinal wall is examined, the same type of metabolism is found as with the isolated mucosa. In fact the metabolism of the whole intestinal wall almost equals the average of the metabolisms of the isolated

Table 5. *Metabolism of mucosa, muscularis and whole wall of the upper part of the jejunum of the rat*

All results calculated on initial dry/wet weight basis.

	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Mucosa	0–20	−11·3	14·8	12·6
	20–40	− 8·7	8·2	5·6
	40–60	− 4·8	3·7	4·0
Muscularis	0–20	− 6·3	4·8	6·8
	20–40	− 4·5	3·2	4·7
	40–60	− 3·9	2·4	4·0
Whole wall	0–20	− 7·8	10·6	8·1
	20–40	− 5·4	8·7	6·6
	40–60	− 3·2	8·0	4·9

mucosa and muscularis (Table 5). In the higher regions of the intestine the metabolism of the whole wall is determined by that of the mucous membrane owing to the higher metabolism and greater weight of the latter. The metabolism of the whole wall of mouse jejunum, especially, greatly resembles that of the isolated mucosa. In the lower regions of the intestine the influence of the muscular coat may be expected to be more marked owing to the decreasing thickness and low metabolism of the mucous membrane.

There is very little loss of weight during an experiment with whole intestinal wall and it therefore does not matter by which method the results are calculated as a comparison of the experiments published in Tables 4 and 6 will show.

Metabolism in serum. According to Negelein [1925] the aerobic glycolysis of normal growing cells such as embryo and the germinal epithelium of testis disappears in serum. This is not the case with intestinal mucosa or whole intestinal wall where aerobic glycolysis persists in serum to at least the same extent as in Ringer solution (Table 6). This result is similar to that obtained previously with the medulla of kidney [Dickens & Weil-Malherbe, 1936, 1] contrary to György *et al.* [1928].

Table 6. *Metabolism of intestine in bicarbonate-Ringer solution and in sheep serum*

All results based on initial dry/wet weight ratio.

		In serum			In Ringer		
	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
Jejunum, mucosa (rat)	0–20	−16·3	25·2	22·9	−10·3	13·3	13·5
	20–40	−15·4	17·7	13·6	− 9·9	11·9	9·8
	40–60	−12·9	14·0	10·6	− 8·4	9·7	8·4
Ileum, mucosa (rat)	0–20	−10·8	8·5	11·7	− 2·1	4·4	7·7
	20–40	− 5·0	3·1	4·7	− 1·2	2·5	5·8
	40–60	− 4·7	1·6	1·4	− 1·6	1·5	4·0
Jejunum, whole wall (mouse)	0–20	−15·5	25·0	10·9	−19·0	25·8	10·6
	20–40	−16·5	20·8	6·6	−15·8	23·2	8·7
	40–60	−14·6	19·2	6·4	−14·8	18·9	7·5

Table 7. *Chemical control of lactic acid formation*

Jejunal mucosa of the rat.

			μl. lactic acid		
Medium*	Gas phase	Time min.	Manometrically	Chemically	% Chemic./manom.
BR	N_2=5% CO_2	120	251	197	78·5
Serum		120	238	206	86·5
Serum		120	460	471	102·5
Serum		150	226	225	100
BR	O_2=5% CO_2	150	560	447	80
BR		150	687	484	70·4
BR		150	643	553	86

Table 8. *Effect of substrate on metabolism of mucous membrane of rat jejunum*

		Q_{O_2}					$Q_G^{N_2}$				
Medium*	Min.	No substrate	0·2% glucose	0·2% glycogen	Q_{CO_2} No substrate	Apparent R.Q.	No substrate	0·2% glucose	0·2% glycogen	0·2% fructose	*M*/100 hexosemonophosphate
					Results based on final dry weight						
BR	0– 30	−30·9	—	—	31·0	1·00	8·7	23·6	22·6	—	—
	30– 60	−14·6	—	—	11·0	0·75	6·3	17·2	16·8	—	—
	60– 90	−12·5	—	—	7·4	0·59	5·0	10·6	11·0	—	—
	90–120	−11·0	—	—	6·8	0·61	5·0	8·2	9·8	—	—
PO_4-R	0– 30	−26·4	−22·2	−21·6	—	—	—	—	—	—	—
	30– 60	−23·7	−22·8	−23·7	—	—	—	—	—	—	—
	60– 90	−19·7	−19·1	−19·4	—	—	—	—	—	—	—
	90–120	−20·8	−20·1	−19·5	—	—	—	—	—	—	—
BR	0– 10	—	—	—	—	—	11·4	48·0	26·1	24·0	13·2
	10– 30	—	—	—	—	—	7·0	27·7	15·2	14·7	12·9
	30– 60	—	—	—	—	—	7·6	16·6	12·8	10·4	12·8
	60– 90	—	—	—	—	—	8·2	9·8	8·2	9·0	8·8
					Results based on initial dry weight						
BR	0– 20	—	—	—	—	—	9·7	9·4	9·8	9·3	9·0
	20– 40	—	—	—	—	—	5·4	6·1	6·2	5·7	4·5
	40– 60	—	—	—	—	—	3·5	5·6	5·0	4·4	3·7
BR	0– 20	—	—	—	—	—	7·3	7·7	11·0	4·7	5·3
	20– 40	—	—	—	—	—	4·6	6·7	5·2	3·9	3·4
	40– 60	—	—	—	—	—	2·6	4·7	3·2	2·7	2·8

* BR = bicarbonate-Ringer solution.
PO_4-R = phosphate-Ringer solution.

Nature of acid formation. That the acid formation by the intestinal mucosa is really due to glycolysis is shown by the fact that 70–100 % of the acid estimated manometrically is accounted for by chemical estimation of lactic acid (Table 7) and secondly by the disappearance of aerobic and, to some extent, anaerobic acid formation in absence of glucose or another suitable substrate (Table 8). Respiration, on the other hand, is not affected at all by the absence of a substrate.

The effect of addition of substrate on the anaerobic glycolysis seems to be variable. In experiments which were performed in early spring we repeatedly observed a strong effect of glucose and glycogen on the anaerobic acid formation which was increased 3–4 times. When we tried to repeat these experiments in November the only effect was a delay in the fall of glycolysis.

Respiratory quotient. For a strongly glycolysing tissue such as the intestinal mucous membrane one might have expected an R.Q. of about unity. In fact the R.Q. is low and decreases towards the ileal end of the jejunum (Table 9).

Table 9. *Respiratory quotient of mucosa from rat jejunum*

Medium*		Time min.	Q_{O_2}	$Q_G^{O_2}$	R.Q.
		Based on final dry weight			
PO_4-R		150	−13·4	—	0·92
PO_4-R		150	−16·6	—	0·89
		100	−28·5	—	0·86
		150	−20·8	—	0·84
		Based on initial dry/wet weight ratio			
BR	Duodenal end	150	− 5·7	7·0	0·93
	Middle part	150	− 5·6	8·6	0·86
	Ileal end	150	−12·5	9·7	0·78
Serum		90	− 5·4	9·1	0·89
		90	− 7·4	10·3	0·83

* BR = bicarbonate-Ringer solution + glucose.
PO_4-R = phosphate-Ringer solution + glucose.

Effect of acetylcholine. In view of the work of Deutsch & Raper [1936; 1938] on the effect of acetylcholine on the *in vitro* metabolism of the salivary glands of the cat it was of interest to examine whether this substance would cause a similar stimulation of metabolism in intestinal mucous membrane, this being largely a glandular tissue controlled by sympathetic and parasympathetic impulses. Experiments were carried out with acetylcholine (1–5μg./ml.), with eserine (20μg./ml.), with atropine (40μg./ml.) and with acetylcholine + eserine. No effect was observed whether the addition was made before or during the experiment.

Nucleic acid-phosphorus. It has been suggested by Berenblum *et al.* [1939, 1] that the measurements of tissue metabolism should be related to the content of nucleic acid rather than to the dry weight. For the conversion of our figures to the nucleic acid standard we have estimated the nucleic acid-P in intestinal mucosa by the method of these authors. We found the following values:

		Nucleic acid-P, % of dry weight
Jejunum:	Proximal part	0·34
	Distal part	0·40
Ileum:	Proximal part	0·44
	Distal part	0·53

Discussion

It is surprising that the exceptional features of the metabolism of intestinal mucous membrane should have been entirely overlooked by previous workers in the field of tissue metabolism. For some reason their attention was turned almost exclusively on the mucosa of the large intestine, yet the experimental difficulties and hence the variation of the results are much less with jejunal mucous membrane which is thicker, less fragile and free from bacteria. It can easily be stripped off from the smooth surface of the small intestine, whereas the surface of the large intestine is undulated owing to ridges of longitudinal muscle fibres which make it difficult to strip off the mucosa without laceration.

The mucous membrane of the jejunum shows the highest metabolic activity of all parts of the intestinal tract. It belongs to the most actively respiring tissues of the body. Its glycolysis in nitrogen is at least of equal activity. Yet this active glycolysis is not depressed by the respiration; it persists at the same rate in oxygen. This complete absence of the Pasteur effect is the most remarkable and indeed unique property of this tissue. There is no reason to assume that the high aerobic glycolysis which we constantly observed is an artefact which does not exist *in vivo*, since it is due neither to the separation from the intestinal wall, nor to incomplete O_2-saturation, nor to bacterial contamination, nor to a toxic effect of saline, nor to a sudden stimulus caused by liberation of acetylcholine.

Although we have never observed a glycolysis which was smaller aerobically than anaerobically in jejunal mucous membrane, the converse was occasionally seen. This was probably due to a more rapid autolysis under anaerobic conditions. It has been shown for brain tissue that there is a rapid fall of the P_2O_7 fraction anaerobically even in presence of glucose with a corresponding fall of glycolysis [Macfarlane & Weil-Malherbe, 1941].

The metabolism of the isolated mucosa of the ileum and colon is low, especially if allowance is made for the disintegration of the tissue during the experiment. Though it was sometimes observed by ourselves as well as by earlier workers that the aerobic glycolysis reached the level of the anaerobic glycolysis, a definite Pasteur effect was found in other experiments. Some of our experiments with colonic mucous membrane agree in fact very well with the figures of Rosenthal & Lasnitzki [1928]. This variation does not seem to recommend the colonic mucous membrane as a suitable subject for the study of the Pasteur effect. Yet it was used for this purpose by Bumm *et al.* [1934] who claimed to have shown that if the O_2-tension is lowered to 10 % the Pasteur effect is inhibited without respiration being affected (cf. Table 1).

As we have previously pointed out [Dickens & Weil-Malherbe, 1936, 1, 2] a high aerobic glycolysis is not specific for either malignant or normal growth. On the other hand a tendency has recently been noticeable [Berenblum *et al.* 1939, 2] to dissociate the high aerobic glycolysis completely from the tumour problem. In experiments to be published one of us has observed examples where there is little change of the metabolism of a normal tissue with low glycolysis during its transformation to a benign tumour or to a precancerous condition, while the development of malignancy is accompanied by a definite increase in the aerobic and anaerobic glycolysis. Thus while aerobic glycolysis is not limited to tumours —and as the present paper shows may even reach the level of the anaerobic glycolysis in a normal 'resting' tissue—it is not correct to assume that the glycolysis of tumour is always merely that of the tissue of origin.

Formerly tumour was considered to be the only tissue where a high glycolysis was accompanied by a low R.Q. We interpreted this [Dickens & Šimer, 1930, 2] as

meaning a defective carbohydrate metabolism, and regarded it as a specific property of tumours. Meanwhile, Bywaters [1937] showed that synovial membrane has a similar type of metabolism, but the low activity of this tissue makes comparison difficult. The mucous membrane of the intestine provides another and more satisfactory example, showing clearly that combination of active glycolysis with low R.Q. is not a specific property of tumours. Another interesting similarity is the occurrence of aminoethylalcohol phosphoric ester in both tumour [Outhouse, 1937] and intestine [Colowick & Cori, 1939].

Summary

The metabolism of mucous membrane from the various parts of the intestine has been measured in Ringer solution and in serum. It is found that the jejunal mucous membrane possesses a high respiration, a high glycolysis, aerobically and anaerobically, and a low R.Q.; acetylcholine was without effect. Jejunal mucous membrane is unique among tissues hitherto studied in that the Pasteur effect is absent. These results are not due to toxic action of Ringer solution, as they are also observed in serum; nor are they due to severance of the mucosa from the matrix as the metabolism of the whole intestinal wall is that expected from the summation of its parts.

The metabolism of mucous membrane from ileum and colon is less active and more variable, probably owing to greater fragility. It resembles in general that of the jejunum, but the Pasteur effect was often evident.

These results are discussed in relation to the problem of tumour metabolism.

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3. THE INFLUENCE OF DIETARY FIBRE ON SECRETORY ACTIVITIES OF THE ALIMENTARY TRACT

OBSERVATIONS ON FAECAL PHOSPHATASE EXCRETION AND CALCIUM AND NITROGEN BALANCES OF RATS

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Some years ago, during an investigation of the influence of rate of growth on the development of rickets in sheep, parallel studies were undertaken using the rat. Owing to the difficulty of obtaining from rats blood samples sufficiently large for phosphatase determination, and having in mind the observations of Heymann [1930; 1931; 1933] on the faecal excretion of phosphatase in rickets, the concentration of this enzyme in the faeces of the rats was determined. It was observed that wide fluctuations occurred in the elimination of phosphatase when animals were transferred from one diet to another. This was especially noticeable in the case of weanling rats transferred to stock diets. The tentative conclusion was drawn that the augmentations in excretion were caused by increased bulk of ingesta which, it was felt, must increase secretory activity within the alimentary tract. The large increases observed for the weanlings were, no doubt, the result of the sensitivity to stimulus of an immature tract accustomed to a diet mainly of milk. These results were not published.

More recently investigations have been in progress in which domestic animals received larger quantities of fibre in the diet than is customary, and certain questions regarding the influence of bulk of ingesta on activities within the alimentary tracts were raised. The connexion between this problem and earlier investigations led to the undertaking of the present study.

Experimental

Six virgin female albino rats aged 105–140 days and weighing 199–225 g. were housed in pairs in three Hopkins metabolism cages which permit of the separate collection of urine and faeces. The animals were fed daily at 10 a.m. after collection of the urine, faeces and unconsumed food. Food and distilled water were offered in excess of appetite. The composition of the low fibre basal diet is recorded in Table 1.

Table 1. *Low fibre diet*

	g.		g.
Sugar	250	$NaHCO_3$	28
Casein	800	$FeSO_4.(NH_4)_2SO_4, 6H_2O$	20
Starch	2200	KH_2PO_4	108
Cottonseed oil	250	NaCl	20
Yeast	160	$MgSO_4, 7H_2O$	20
$CaCO_3$	100	Radiostoleum	4 ml.
KCl	40		

The experiment was divided into five periods, the first of 10 days' duration and the remainder of 6 days each. During period 1 the basal diet was offered. During period 2 the basal diet was supplemented with 10% of acid-washed Ca-free paper pulp. In period 3 20% and in period 4 30% of paper pulp was added. Period 5, in which 40% of paper pulp was added to the basal diet, ended abruptly owing to failure of appetite. The day's ration was made up in a stiff dough to minimize scattering and selective feeding.

There were no intervals for adjustment between the periods, the balances being continuous.

Methods of analysis

Calcium. Samples of urine and faeces were dried and ignited at a dull red heat until all traces of carbon had disappeared. The ash was dissolved in hot dilute HCl and made up to known volume. Aliquots were transferred to conical centrifuge tubes, made just alkaline with dilute NH_4OH and then just acid with dilute CH_3COOH using methyl red as an indicator. The tubes were placed in a boiling water bath and, after a few minutes, an excess of a boiling solution of 4% $(COONH_4)_2$ was added. The tubes were then held at about 70° for 1 hr. to induce the formation of granular precipitates and allowed to stand at room temperature overnight. The precipitates were centrifuged and washed with 2% NH_4OH. Supernatant liquors were removed by siphoning to avoid loss of precipitate. The oxalate, dissolved in dilute H_2SO_4, was titrated with permanganate.

Nitrogen. All estimations were performed by the macro-Kjeldahl method.

Phosphatase. The day's faeces were well mixed and a sample taken. The sample was placed in a bottle with 200 ml. distilled water and a little toluene and shaken in an end-over-end shaker for about 4 hr. by which time disintegration was complete. The sample was left at room temperature overnight and then analysed according to Bodansky's [1933] method for serum phosphatase. All results are expressed as Bodansky units.

Results

The Ca balances are recorded in Table 2 and the N balances in Table 3. In Table 4 are given the daily phosphatase excretions in the faeces and the units of phosphatase per g. of fresh faeces. All values given are for the group as a whole.

The animals remained in good health throughout the experiment. During period 1 no diarrhoea occurred but occasionally large, moist boli were passed. Throughout the experiment body weight increased from a mean value of 203 g. at the beginning to a mean value of 229 g. at the end. A certain amount of this increase can be attributed to greater weight of intestinal contents during the periods of high fibre intake.

The inclusion of increasing amounts of fibre caused progressive reductions in the intakes of basal ration. These reductions were moderate, the addition of the maximal quantity of fibre (30% in period 4) causing a fall of about 20% in the basal diet intake from the period 1 level.

Urinary Ca values were low, only about 1% of ingested Ca being excreted by the kidneys. There were slight erratic variations in output, but the range was not wide and the mean excretions for each period were unrelated to changes in fibre intake. The major portion of the Ca was found in the faeces. The balances were positive throughout, the best retention occurring during the period when 10% of fibre was included in the diet. In periods 3 and 4 the retentions were about the same and, in magnitude, about two-thirds of the period 2 retention.

Table 2. *The effect of adding to the diet increasing amounts of fibre on the daily Ca balances of rats*

Period	Day	Ca intake mg.	Ca output mg.	Ca balance mg.	Ca in urine mg.	Ca in faeces mg.
1	1	1040	1003	+ 37	10·7	992
	5	983	867	+116	6·3	861
	6	1026	1081	− 55	5·4	1076
	7	1004	921	+ 83	7·6	913
	8	1000	922	+ 78	8·4	914
	9	971	982	− 11	6·7	975
	10	913	939	− 26	6·2	933
2	1	960	961	− 1	7·1	954
	2	960	—	—	—	—
	3	1074	953	+121	7·4	946
	4	1002	888	+114	8·2	880
	5	944	888	+ 56	9·3	879
	6	880	835	+ 45	7·1	828
3	1	851	839	+ 12	6·6	832
	2	827	788	+ 39	5·6	782
	3	848	817	+ 31	5·3	812
	4	814	771	+ 43	5·4	766
	5	829	788	+ 41	9·7	778
	6	913	799	+114	7·9	791
4	1	794	853	− 59	6·1	847
	2	795	707	+ 88	5·5	702
	3	815	712	+103	6·9	705
	4	731	697	+ 34	6·4	691
	5	836	782	+ 54	7·9	774
	6	755	723	+ 32	11·4	712
			Averages			
1	—	991	959	+ 32	7·3	952
2	—	972	905	+ 67	7·8	897
3	—	847	800	+ 47	6·8	794
4	—	788	746	+ 42	7·4	738

Urinary N values declined from period to period as the N intake fell. The faecal excretion increased from period 1 to period 2 and again in period 3. The value found for period 4 was somewhat below the period 2 level. The mean retentions were positive in all periods, higher retentions being found in periods 1 and 3 and the lowest in period 4. Negative daily balances preponderated in period 4 but were absent in period 1. Occasional negative daily balances occurred during periods 2 and 3.

During period 1 there were moderate fluctuations in the daily excretion of phosphatase but, although the range was wider than might have been expected, the inclusion of 10 % of fibre in the diet caused a large increase in excretion well beyond the upper limit of period 1 range. In period 3 the excretion of phosphatase was again greater than in period 2 and the values for period 4 were higher than in period 3. The increases with each addition of fibre were progressively less. The mean values showed a more than sevenfold increase between periods 1 and 4. The units of phosphatase excreted per g. of fresh faeces increased in periods 2 and 3 over period 1, but in period 4 the value fell to a point midway between the two extremes. In each of the fibre periods the sudden increase in fibre intake led to maxima in faecal phosphatase excretion, after which the animals tended to adjust themselves to the new conditions.

Table 3. *The effect of adding to the diet increasing amounts of fibre on the daily N balances of rats*

Period	Day	N intake mg.	N output mg.	N balance mg.	N in urine mg.	N in faeces mg.
1	1	3016	2745	+271	2392	353
	5	2852	2544	+308	2260	284
	6	2979	2720	+257	2400	320
	7	2914	2806	+108	2504	302
	8	2901	2660	+241	2350	310
	9	2930	2655	+275	2349	306
	10	2654	2604	+ 50	2328	276
2	1	2743	2436	+307	2092	344
	2	2744	2607	+137	2264	343
	3	3101	3015	+ 86	2665	350
	4	2876	2563	+313	2250	313
	5	2513	2634	−121	2278	356
	6	2312	2575	−263	2272	303
3	1	2458	2223	+235	1738	485
	2	2388	2430	− 42	2090	340
	3	2450	2242	+208	1900	342
	4	2391	2289	+ 62	1990	299
	5	2393	2216	+177	1822	394
	6	2640	2464	+176	2130	334
4	1	2281	2292	− 11	1950	342
	2	2286	2188	+ 98	1839	349
	3	2347	2387	− 40	2062	325
	4	2095	2155	− 60	1870	285
	5	2408	2213	+195	1906	307
	6	2166	2213	− 47	1874	339
			Averages			
1	—	2891	2676	+215	2369	307
2	—	2715	2638	+ 77	2303	335
3	—	2447	2311	+136	1945	366
4	—	2264	2241	+ 23	1917	324

Table 4. *The effect of adding to the diet increasing amounts of fibre on the daily faecal excretion of phosphatase by rats*

Period	Day	Total faecal phosphatase	Phosphatase per g. of faeces	Period	Day	Total faecal phosphatase	Phosphatase per g. of faeces
1	1	182	14·4	3	1	1061	24·2
	5	149	15·0		2	1178	27·4
	6	186	15·4		3	1433	29·9
	7	165	15·6		4	1230	29·0
	8	239	22·0		5	1286	27·8
	9	292	24·6		6	1074	24·8
	10	200	18·5				
Mean		202	17·9	Mean		1210	27·7
2	1	637	28·5	4	1	1598	23·4
	2	1033	33·7		2	1589	22·3
	3	1010	31·5		3	1436	20·8
	4	676	24·4		4	1423	22·0
	5	870	29·0		5	1513	22·2
	6	535	22·9		6	1306	19·3
Mean		794	28·3	Mean		1478	21·7

Discussion

It is felt that the preliminary nature of the observations of the effect of large quantities of crude fibre in the diet on phosphatase excretion in the faeces should be emphasized. At the present time an exact interpretation of the results is not possible because neither the individual contribution of the different secreting organs nor the rates of destruction of the secreted enzyme within the lumen of the intestine during the different periods were assessed. Nevertheless certain conclusions can profitably be drawn.

While it is possible that there was a proportional increase of all secretions, amounting to about 700 % in period 4 as compared with period 1, it is more probable that there were greater increases in the secretions of certain organs and smaller, or even no, increases in the case of others. Also, there is no information regarding the possible exhaustion, or semi-exhaustion, of the enzyme within an organ suddenly called upon to increase secretion. In such a case the phosphatase value of the faeces would be, by underestimation, an imperfect guide to measurement of increased secretory activity. Although there is no clear evidence that bulk of ingesta has any marked effect in increasing the speed of propulsion of material through the intestinal tract, if this does occur the faecal phosphatase value would overestimate increased secretory activity owing to reduced opportunity for enzyme destruction.

In the present investigation an objection might be raised to comparing period 1 with period 4 on the ground that this involves the assumption that results found for the fibre-free period represent the normal state of affairs and can therefore be accepted as basic in subsequent discussion. But even if this period be discarded and period 2, with the 10 % fibre intake, be accepted as normal, period 4 still exhibits an increase of 100 % in faecal phosphatase excretion. If, however, the balance data for Ca and N can be taken as a guide there is no evidence of dysfunction during period 1. Moreover, beyond the occasional appearance of moist boli, defecation was normal.

The main source of the increases in faecal phosphatase excretion is probably the wall of the intestine, since the greater bulk calls for more lubrication during propulsion. In this connexion the distribution of phosphatase within the faecal boli was examined and it was found that the concentration was greater in the pellicle than in the centre. This difference could only have arisen after the boli had been formed and was therefore attributable to the secretory activity of the large intestine. During the periods when 10 and 20 % of fibre were added to the diet the quantity of phosphatase per g. of faeces fell within the same range for both periods and the average values were closely similar. In period 4, which represents with an intake of 30 % of fibre about the limit that the rat will tolerate, the excretion per g. of faeces fell. This coincides with the concentration of dietary fibre which Westerlund [1938, 2] found increased the faecal Ca output in the rat when fed on a very low Ca diet. The phosphatase data suggest that a 30 % level of fibre reduces the efficiency of the intestine in relation to the bulk of ingesta although the total activity is further increased (from the period 2 level). This impairment of efficiency is also revealed in Westerlund's experiment, where Ca intake was very low. As is discussed below this does not occur with diets of normal Ca content.

Several workers have studied the influence of dietary fibre on Ca retention. Bloom [1930] found that the addition of 8 % of fibre to a diet of normal Ca content had no effect upon Ca retention of rats, and this was confirmed by Adolph *et al.* [1938] who, however, used a low Ca diet. Ascham [1930–31] considered that bulk

may increase faecal Ca losses in dogs but felt that her results might be inconclusive. Conversely, Westerlund [1938, 1, 2] reported that the incorporation of paper pulp or acetone-extracted, electrodialysed straw flour beyond the level of 20% increased the faecal elimination of Ca by rats subsisting on a diet extremely low in Ca and P. It was considered that the increased bulk stimulated secretory activities within the intestine and that an increased rate of transit of the ingesta through the intestine prevented complete reabsorption of the Ca of the secretions, and the conclusion was drawn that Ca requirement was increased. As stated above, there is no good evidence that increased bulk promotes more rapid propulsion of ingesta, and Westerlund states that he had previously failed to establish any relation between Ca absorption and crude fibre consumption in dairy cows. But, as is discussed below, faecal Ca studies and balance data cannot measure total absorptive processes; only net values can be derived. Morgan [1934], studying adult human subjects consuming coarsely ground cellulose added to a standard diet, found that Ca balances tended to become negative during the high fibre period. Sjollema [1923] observed that increased fibre consumption resulted in negative Ca balances in the rabbit where balances previously had been positive. It is generally accepted that the rabbit is an unsatisfactory animal for Ca balance studies owing to the erratic nature of the results yielded. This peculiarity of the rabbit is well shown by the wide variations in blood Ca frequently observed and by the observation of Cowell [1937] that the rabbit excretes considerable quantities of Ca through the colon. This last characteristic is the opposite of that found by Nicolaysen [1934] and Christiansen [1936] for dogs and by McCance & Widdowson [1939] for man.

The Ca balance results of the present study agree with those of Bloom [1930] and of Adolph *et al.* [1938]. But the results can only be adequately interpreted when examined in conjunction with the phosphatase values. Although it was reasonable to expect that increased bulk of ingesta would induce greater secretory activity within the alimentary tract, it was not expected that such increase would be sevenfold. It is remarkable that such an increase was not associated with a detrimental effect upon the Ca balance. In this respect it is of importance to consider the observation of Nicolaysen [1934] that the output of Ca in the digestive juices of adult man amounts to about 1 g. daily, that is to say, about twice the daily Ca requirement. (This observation is similar to that of Watson [1933] who observed that the salivary P, mainly inorganic, of sheep can amount to as much as 7·7 g. daily, which is several times the daily requirement for growth.) That reabsorption of this secreted Ca is conducted with an extraordinary degree of efficiency is made clear by considering the phosphatase increases. The enormous increase in secretory activity which must have been associated with the increases observed in faecal phosphatase occasioned no losses of Ca from the organism. It is probably correct to assume that the increase in the volume of the secretions would cause some diminution in the concentration of Ca in the secretions, but it is unlikely that the reduction would be severe, since the electrolyte pattern of the secretions bears a relation to that of the blood (as has been shown by Watson for salivary P and blood P in sheep) and there is no record of increased bulk of ingesta reducing blood Ca.

The efficiency with which the secreted Ca is reabsorbed is probably the outcome of the Ca being in ionized form, or at least in forms readily ionizable under conditions existing within the intestine.

Mitchell [1923–24] found that N excretion was increased by 42% when rats receiving a N-free, low fibre diet had access to filter paper and his 'metabolic N' correction is generally accepted in certain types of protein metabolism studies.

Whiteacre *et al.* [1929–30] considered that high fibre diets reduced the N balances in human subjects but, unfortunately, no analyses of the diets for crude fibre were performed. Morgan [1934] reported that an increased fibre intake had a slight detrimental effect on N retention in man. In the case of the rabbit, Sjollema [1923] failed to find any effect of crude fibre on N retention.

The generally observed detrimental effects on N retention of increased fibre intake were found in the present study. The highest retention was observed during the period of lowest fibre intake and the lowest retention occurred during the period of highest fibre intake. During period 3 a higher retention was observed than in period 2; this may have arisen owing to the shortness of the experimental periods and the lack of accustoming intervals between periods. The percentage of N intake excreted in the faeces for the 0, 10, 20 and 30 % fibre periods were: 10·6, 12·3, 15·0 and 14·4. In this case again an unexpectedly high value occurs in period 3, resulting from the aberrant values found for urinary and faecal N on the first day of this period when, apparently, there was poor absorption of N. If the faecal N value for this day is omitted in calculating the percentage of dietary N lost in the faeces the series becomes: 10·6, 12·3, 14·0 and 14·4. Since the faecal N originates only in part from the dietary N, an attempt is made in Table 5 to recalculate the data in such a manner as to reveal any connexion existing between the increases in faecal N excretion consequent on increased fibre intakes and increased phosphate excretions. It is realized that several debatable assumptions are involved in this approach but, in the absence of values for the different forms of N in the faeces, a first approximation is attained.

Table 5. *Relation of 'N-cost' of dietary fibre to the excretion of phosphatase in faeces*

Period	Faecal N mg.	N originating from intake mg.	'N-cost' mg.	Phosphatase increase	Phosphatase increase / 'N-cost'
1	307	—	—	—	—
2	335	288	47	592	13
3	342	259	83	1008	12
4	324	240	84	1276	15

In column 2 are recorded the faecal N averages from Table 3 (except in the case of period 3, where the aberrant value for the first day has been excluded from the calculation of the average). In column 3 is recorded the quantity of N in the faeces that is calculated to have arisen from and on account of the basal diet intake, the computation being based on the period 1 finding that the faecal N is equal to 10·6 % of N intake when no fibre is added to the diet. As stated above, Mitchell found that the 'metabolic-N' bore a close relation to the quantity of diet consumed; this was confirmed by Adolph & Wu [1934], who demonstrated in addition that increased bulk of ingesta did not interfere with corrected protein digestibility unless the material passed through the intestine at a more rapid rate than usual. Neither Adolph & Wu nor ourselves found any evidence of increased rate of transit when filter paper was fed to rats. In column 4 are given the differences between the values in columns 2 and 3; this fraction is considered to be the portion of the faecal N which was lost to the animal as a result of the quantity of fibre ingested and is referred to as the 'N-cost' of the fraction. In column 5 are given the increases in phosphatase excretion over the basal period 1 level. Finally, in the last column are presented the ratios Phosphatase increase: 'N-cost'.

The agreement between the ratios suggests that there is a relation between phosphatase excretion and increased N excretion on higher fibre intakes. Theoretically, it seems probable that the extra N eliminated is partly in the form of epithelial fragments and partly mucoprotein. It is to be expected that such contributions of these forms of N as occur in the lower regions of the small intestine will escape hydrolysis and absorption. It will certainly be true of those originating in the large intestine.

From the results obtained it is indicated that studies of phosphatase excretion may be valuable aids in investigations of secretory activities of the intestinal tract and this probably applies also in the case of other enzymes. In questions of pathological changes of the tract the estimation might be of value. Concerning the latter, the test might be of use in studies of cases of hypersensitivity of the intestinal tract to fibre in human subjects.

Summary

Increasing the fibre content of the diet of rats to levels as high as 30% had no influence on Ca retention but reduced the N balance.

Large increases were observed in faecal phosphatase excretion when the fibre intake was raised and this was thought to indicate increased secretory activity within the intestines.

The absence of any depressing effect of large increases in the intestinal secretions on the Ca balance was considered to indicate that the reabsorption of secreted Ca is highly efficient.

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4. THE COMPONENT ACIDS OF PHOSPHATIDES PRESENT IN COW'S MILK FAT

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Although until quite recently phosphatides were considered to play an important part in the production of the characteristic glycerides present in milk, or even to be the precursors of the latter, a quantitative statement of the composition of the mixed fatty acids in milk fat phosphatides was lacking until the fatty acids of phosphatides from butter milk powder were examined by Kurtz *et al.* [1934]. Previously, however, Osborne & Wakeman [1915] had reported the presence of saturated acids of M.P. 62–65° and of liquid acids of iodine value 85·7 in phosphatides isolated from cow's milk, whilst Sasaki & Hiratzuka [1931], who had prepared purified phosphatides from dried milk, observed lauric, myristic and palmitic acids amongst the fatty acids present, but found no evidence of the presence of unsaturated acids. Kurtz *et al.* [1934] extracted the phospholipids from 300 lb. of buttermilk powder with a mixture of 75% ethyl alcohol and 25% of light petroleum and separated the material precipitated from acetone into two fractions, (*a*) phosphatides soluble in ether at 0° and (*b*) substances insoluble in this medium which appeared to be of the nature of sphingomyelin and cerebrosides (*vide infra*, pp. 27, 29). The phosphatides of fraction (*a*) yielded on hydrolysis 62% of fatty acids, the component acids of which were observed to be myristic 5·2, stearic 16·1, arachidic 1·8, oleic 70·6 and dicostetraenoic 6·3% (wt.). These authors were unable to detect any palmitic acid, but identified myristic, stearic and arachidic acids in the ester fractions which they obtained; of unsaturated acids, they established the presence of oleic acid in quantity, and also of a highly unsaturated acid of higher molecular weight for which the analytical data indicated a formula $C_{22}H_{36}O_2$, but found no evidence for di- or tri-enoic C_{18} acids. They also found that, in contrast to butter fat, the phosphatides contained none of the lower saturated fatty acids.

We have recently examined the acids from two specimens of milk fat phosphatides, but the quantities of each in our possession did not permit us to separate the 'lecithin-kephalin' fraction from other compounds also present, possibly of the types noted by Kurtz *et al.* [1934] or possibly also including a certain proportion of wax-esters. We have, however, submitted the mixed acids from each specimen to analysis by ester fractionation and have confirmed the findings of other workers that the typical lower saturated acids of cow's milk fat are wholly absent. Our data, like those of Kurtz *et al.* [1934], disclose the presence of up to 10% of highly unsaturated acids of molecular weight corresponding to members of the C_{20} and C_{22} series (in common with all animal phosphatide component acids so far studied). Our experience differs, however, from that of Kurtz *et al.* [1934] in that, amongst the saturated acids, we observed the presence of relatively large proportions of palmitic acid.

Experimental

The first specimen of milk fat phosphatides which we studied was kindly placed at our disposal by Dr B. Rewald, who had isolated it from non-glyceridic residues obtained in the separation of pure butter fat from Swiss butter. These had been extracted, first with light petroleum and then with a mixture of 80% alcohol and 20% benzene, the extract from this second operation being subsequently re-extracted with light petroleum. The combined light petroleum extracts were boiled with acetone and the portion which separated in the cold was the subject of the present work (the material left in solution in acetone was found to be mainly butter fat). In consequence of the differences between the results we obtained with this specimen and those of Kurtz *et al.* [1934] we proceeded to isolate, employing the procedure of Rewald [1937], the corresponding phosphatide fraction from residues from English butter fat clarification kindly provided for us by the Pure Butter Fat Process Co.

Component acids of the phosphatides from Swiss butter residues

The material received from Dr Rewald consisted of friable, dark brown, somewhat hygroscopic granules; it had a saponification equivalent of 221·4 and gave on hydrolysis mixed fatty acids which had (after removal of unsaponifiable matter) sap. equiv. 290·2, I.V. 37·0.

The corresponding material left in solution in acetone in the later stages of Dr Rewald's isolation of the butter phosphatides had sap. equiv. 243·5, I.V. 38·4. We submitted a portion (111 g.) to complete analysis by the usual fractionation procedure and found its component acids to be butyric 3·7, *n*-hexanoic 1·8, *n*-octanoic 1·2, *n*-decanoic 2·4, lauric 2·6, myristic 11·8, palmitic 28·6, stearic 8·9, arachidic 1·2%; decenoic 0·2, dodecenoic 0·4, tetradecenoic 1·0, hexadecenoic 2·8, oleic 27·3, octadecadienoic 5·8, unsaturated C_{20-22} 0·3% (wt.). It thus evidently consisted of typical butter fat glycerides.

The Swiss butter phosphatides (40·7 g.) were hydrolysed, first with 0·5% alcoholic H_2SO_4 (500 ml.) and subsequently with alcoholic KOH. After thorough removal of alcohol from the soaps produced, the latter were dissolved in water, acidified with H_2SO_4 and steam-distilled for 5 hr. The steam-distillate was exhaustively extracted with ether and the ether-extracted aqueous portion (A) and the ether distilled from the extract (E) were examined for acidity. The material extracted by ether (0·57 g., equivalent 229·3) was solid at room temperature; no odour of butyric or other fatty acids of low molecular weight was, moreover, perceptible in the original aqueous condensate from the steam distillation. Both the aqueous portion (A) and the recovered ether (E) nevertheless possessed definite acidity, but this was shown to be due mainly to HCl and, to a less degree, to traces of sulphurous and/or sulphuric acid. Aliquot parts of (A) and (E) were exactly neutralized, evaporated to a small bulk, and any chloride estimated as silver chloride; another aliquot portion of (A) was treated with H_2O_2 in the cold, then concentrated to a small volume, and any H_2SO_4 was determined as $BaSO_4$; a control experiment carried out *ab initio* with the alcohol used in the hydrolysis of the phosphatides served to account for any traces of volatile acids emanating from the alcohol. The results of these determinations are summarized on the following page.

The observed acidity was therefore mainly due to HCl, presumably from NaCl carried down with the phosphatides in the course of their extraction.

Butyric or other low molecular weight acids, present in butter glycerides, were totally absent from the phosphatides.

		Equivalents of acid present
(A) Aqueous steam-distillate after extraction with ether:		
Total acidity by titration		0·02482
Volatile acids from alcohol (control experiment)	0·00023	
Acidity due to hydrochloric acid	0·02184	
Acidity due to sulphurous or sulphuric acid	0·00263	
Total accounted for		0·02470
(E) Ether from ether-extract of steam-distillates:		
Total acidity by titration		0·00098
Acidity due to hydrochloric acid		0·00032

The acids non-volatile in steam were recovered and submitted to lead salt separation. The total fatty matter accounted for was as follows:

	g.	%
Steam-volatile fatty acids	0·57	3·2
Non-steam-volatile fatty acids:		
'Solid' acids S	8·32	46·2
'Liquid' acids L	8·63	48·0
Unsaponifiable matter	0·47	2·6
	17·99	

The yield of fatty acids from the phosphatides (44 %) was low, in consequence of the fact that the small amount of material available precluded further purification of the 'lecithin-kephalin' fraction.

The 'solid' and 'liquid' acids were separately converted into methyl esters and the latter distilled *in vacuo* through an electrically heated and packed fractionating column; the fractions listed in Table 1 were obtained.

Table 1

No.	g.	B.P./0·1 mm.	Sap. equiv.	I.V.
		(i) Methyl esters of 'solid' acids S		
S1	2·53	80–100°	270·7	4·2
S2	1·86	100°	290·2	12·8
S3*	2·40	Residue in column	318·7	—
S4*	1·40	Residue in distilling-bulb	368·9	—
	8·19			
		(ii) Methyl esters of 'liquid' acids L		
L1	2·42	100–120°	279·0	70·6
L2	3·05	120–126°	297·2	103·3
L3	2·28	Residue in column	301·9	109·2
L4*	0·92	Residue in distilling-bulb	448·6	—
	8·67			

* Esters, freed from unsaponifiable matter: S3, sap. equiv. 317·5, I.V. 17·3; S4, sap. equiv. 366·4, I.V. 11·7; L4, sap. equiv. 354·0, I.V. 90·4.

The following acids were identified in certain of the above fractions:

Acids volatile in steam (0·57 g., equiv. 229·3). Crystallization from 90 % alcohol gave a crystalline fraction, M.P. 56° (mixed with palmitic acid, M.P. 58–60°): this appeared to be a mixture of palmitic and myristic acids.

'Solid' acids S. The acids from fraction S1, on crystallization from 90 % alcohol followed by ethyl acetate, gave palmitic acid (M.P. 61·0°, mixed with pure

palmitic acid M.P. 62·0°). Those from fraction S3, after four crystallizations from ethyl acetate, yielded pure arachidic acid (M.P., and mixed M.P., 74–74·5°). Those from the residue S4 could not be obtained pure, but gave, from ethyl acetate, a mixture of acids which melted at 76·5° (mixed with pure arachidic acid, M.P. 71–72°); acids of higher molecular weight than arachidic acid were obviously present.

'Liquid' acids L. The acids from fraction L3 were dissolved in anhydrous ether at 0° and treated with bromine; insoluble bromo-additive compounds were precipitated, which commenced to darken at 200–210° and decomposed without completely melting at 224° (characteristic for highly unsaturated C_{20-22} acids).

The presence of arachidic acid is satisfactorily established, but the nature of the saturated acids of higher molecular weight remains uncertain. These may represent wax esters present in traces in the original milk fat and concentrated by the process of extraction in the phosphatide (acetone-insoluble) fraction; or, perhaps more probably in the light of recent work by Kurtz & Holm [1939], they may emanate from bodies of a sphingomyelin or cerebroside nature. In the former case they would be mainly of the formula $C_{26}H_{52}O_2$, in the latter $C_{24}H_{48}O_2$. For the purpose of the present calculations they have been taken as $C_{26}H_{52}O_2$. The final composition of the mixed fatty acids from the Swiss butter phosphatide concentrate is then as in Table 2 (the precision of the analyses is not considered to be of the order attainable with more adequate amounts of experimental material).

Table 2. *Component acids of phosphatides from Swiss butter residues (approx.)*

		Acids non-volatile in steam					
Acid	Steam volatile acids (3·2%)	'Solid' acids S (46·2%)	'Liquid' acids L (48·0%)	Unsaponifiable (2·6%)	Total %	% (wt.)*	% (mol.)*
Myristic	3·03	0·06	—	—	3·09	3·2	4·0
Palmitic	0·17	16·20	3·85	—	20·22	21·0	23·2
Stearic	—	6·99	—	—	6·99	7·3	7·2
As arachidic	—	11·83	—	—	11·83	12·3	11·1
As $C_{26}H_{52}O_2$	—	4·97	—	—	4·97	5·2	3·7
Hexadecenoic	—	—	4·13	—	4·13	4·3	4·8
Oleic	—	6·03	25·22	—	31·25	32·5	32·5
As octadecadienoic	—	—	6·19	—	6·19	6·4	6·5
As C_{20-22} unsatd.	—	—	7·51	—	7·51	7·8	7·0
Unsaponifiable	—	0·12	1·10	2·60	3·82	—	—

* Excluding unsaponifiable matter.

Component acids of the phosphatides from English butter residues

In the hope of obtaining a larger quantity of material for examination, attempts were made to find a suitable source of the milk fat phosphatide fraction in this country. Although, ultimately, a specimen of no larger size than that discussed above was obtained, the results are here included since the fatty acid composition arrived at differed in some details from that of the preceding sample.

In the first place, preliminary experiments on three possible sources of milk fat phosphatides were carried out, namely, on buttermilk powder and on two varieties of residues from butter fat clarification processes. We are indebted to the Wilts United Dairies Ltd. for the buttermilk powder, and to the Pure Butter Fat Process Company who were good enough to supply us with the other two samples. Small quantities of each product were refluxed with ether and the

solutions filtered. The matter soluble in ether was recovered and extracted with light petroleum, and the portion passing into solution was recovered and boiled with acetone. The percentages of acetone-insoluble products (i.e. phosphatide concentrates) from the three specimens were:

	Phosphatide concentrate %
Buttermilk powder	0·4
Butter fat residue A	0·7
Butter fat residue B	2·9

The residue A was a thick yellow emulsion of the consistency of cream, whilst residue B was a dark brown semi-solid paste. In consequence of its much higher apparent phosphatide content, the butter fat residue B was selected as the source of larger supplies of phosphatides and about 8 lb. of the material were extracted with ether. The extracted material was treated first with light petroleum and later with a mixture of 80 % of ethyl alcohol and 20 % of benzene, and the crude phosphatides obtained were separated from acetone as previously described. Ultimately, however, only about 42 g. of the purified phosphatides were recovered for further study. (The acetone-soluble matter extracted from the final phosphatide concentrates had sap. equiv. 246·6, I.V. 40·6, and therefore, as in the preceding instance (p. 25), probably consisted mainly of ordinary butter fat glycerides.)

The phosphatide concentrates (38·5 g.) gave on hydrolysis 15·8 g. of fatty acids (with some unsaponifiable matter); the yield of fatty acids was low, as in the case of the Swiss butter phosphatides. After removal of alcohol from the saponified product, followed by acidification with H_2SO_4, no odour of butyric or other low molecular weight acid could be detected, and the distillation of the mixed fatty acids with steam was accordingly omitted. On submission to a lead salt-alcohol separation the mixed acids (15·82 g.) gave 'solid' acids (9·28 g., 58·7 %), 'liquid' acids (5·49 g., 34·7 %) and a small amount of non-fatty material insoluble in ether (1·05 g., 6·6 %). Distillation at 0·1 mm. of the methyl esters of the two groups of acids through the fractionating column gave a series of fractions, and from the analyses of the latter the approximate composition of the mixed fatty acids was found to be as given in Table 3.

Table 3. *Component acids of phosphatides from English butter residues (approx.)*

Acid	'Solid' acids (58·7 %)	'Liquid' acids (34·7 %)	Unsaponifiable (6·6 %)	Total %	% (wt.)*	% (mol.)*
Myristic	4·74	—	—	4·74	5·5	7·0
Palmitic	7·74	3·89	—	11·63	13·4	15·2
Stearic	7·85	—	—	7·85	9·0	9·2
As arachidic	18·18	—	—	18·18	20·9	19·5
As $C_{26}H_{52}O_2$	8·70	—	—	8·70	10·0	7·3
Hexadecenoic	—	4·30	—	4·30	4·9	5·7
As oleic	9·96	10·49	—	20·45	23·5	24·3
As C_{20-22} unsaturated	—	11·11	—	11·11	12·8	11·8
Unsaponifiable	1·53	4·91	6·60	13·04	—	—

* Excluding unsaponifiable matter.

The component acids in this sample of butter phosphatides are of the same general type as those in the Swiss phosphatides, but contain more of the higher saturated acids, i.e. a higher proportion of admixed wax esters, or

sphingomyelin and cerebrosides, as the case may be. When allowance is made for this, however, it appears that the palmitic and oleic acid contents disclosed by this analysis are somewhat lower than in the preceding case. Acids of the C_{18} group more unsaturated than oleic were not definitely detected, but the small amount of methyl esters of 'liquid' acids available for fractionation may well have been responsible for this.

Discussion and summary

The results of the two analyses of cow's milk phosphatide component acids described above fully confirm the findings of previous investigators that the typical milk fat glyceride acids (butyric, etc.) of low molecular weight are wholly absent from the phosphatide acids. It has been demonstrated [Graham *et al.* 1936; Maynard *et al.* 1938] that the blood phosphatides of the cow are not removed by the mammary gland, and that these are not the precursors of milk fat glycerides. It is now equally well established that the component fatty acids of milk fat phosphatides have little in common with those of the milk fat glycerides; on the other hand, they bear more general similarity to those of the phosphatides of the ox liver [Hilditch & Shorland, 1937], as the following comparison indicates:

Table 4. *Component acids of ox liver and cow's milk phosphatides*

Phosphatides of ...	Ox liver [Hilditch & Shorland, 1937]	Cow's milk fat (Present work)		Kurtz *et al.* [1934]
		'Swiss'	'English'	
Acid	% (wt.)	% (wt.)	% (wt.)	% (wt.)
Myristic	1·3	3·2	5·5	5·2
Palmitic	28·2	21·0	13·4	—
Stearic	14·4	7·3	9·0	16·1
As arachidic	0·2	12·3	20·9	1·8
As $C_{26}H_{52}O_2$	—	5·2	10·0	—
Tetradecenoic	0·7	—	—	—
Hexadecenoic	3·8	4·3	4·9	—
Oleic	17·3	32·5	23·5	70·6
As octadecadienoic	14·2	6·4	—	—
As C_{20-22} unsaturated	19·9	7·8	12·8	6·3

So far as our analyses are concerned, the points of resemblance with the liver phosphatide component acids are the presence, as major components, of palmitic and oleic acids, together with notable proportions of stearic and of highly unsaturated acids of the C_{20} and C_{22} series; the chief points of difference are the minor proportions of octadecadienoic acid in the milk fat phosphatides, and the presence of considerable amounts of arachidic acid and of higher saturated acids (reckoned arbitrarily as $C_{26}H_{52}O_2$). The compounds giving rise to most of these higher saturated acids were separated, by Kurtz *et al.* [1934], by crystallizing the phosphatide concentrates from ether at 0°. Kurtz & Holm [1939] state that the ether-insoluble fraction contains about 80 % of lignoceric acid, the remaining 20 % being a very complex mixture of saturated, unsaturated, hydroxy-saturated and presumably hydroxy-unsaturated acids, the carbohydrate, phosphorus and nitrogen contents of this fraction supporting the view that it is a mixture of sphingomyelin and cerebrosides. It is possible that simultaneous separation from ether of some of the more saturated 'lecithin-kephalin' phosphatides is

responsible for the large proportions of oleic acid present in the ether-soluble fraction the composition of which was given by Kurtz *et al.* [1934]; but their failure to detect any palmitic acid in either group of the phosphatides renders the composition of the material which they examined unusual, if not unique, in the records of component acids of phosphatides from any source.

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5. PALM OIL CAROTENOIDS

1. THE ISOLATION OF LIPOID PIGMENTS FROM A 'SHERBRO' PALM OIL

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The colour of red African palm oils is generally attributed in the main to the presence of carotene, a relatively high proportion of which is assumed to occur in the form of the optically active α-isomer [Kuhn & Brockmann, 1931]. Actually, qualitative chromatographic examination of the unsaponifiable matter of typical palm oils provides immediate evidence of the multiplicity of the coloured constituents exhibiting the usual carotenoid characteristics. It is to be expected that the nature of the carotenoids present in palm oils will vary to some extent with the district of their origin and their previous history. It therefore appeared to us that an examination of some typical palm oils might prove of interest from the point of view of identifying known carotenoids and throwing new light on the problem of their biogenesis; moreover, such investigations might furnish examples of other members of the group which have not previously been detected in nature. In this paper, which is to be regarded as of an introductory character, attention has been directed to an investigation of the carotenoids present in 'Sherbro' palm oil.

With the object of minimizing as far as possible any tendency for the carotenoids to undergo isomeric changes of the type recently described by Zechmeister & Tuzson [1938], and by Carter & Gillam [1939], the saponification of the oil was accomplished at as low a temperature as reasonably possible. Extraction with light petroleum and thereafter with methylated ether, followed by evaporation under reduced pressure in an atmosphere of CO_2, furnished fractions which may be described for the sake of convenience as petrol-soluble unsaponifiable matter and ether-soluble unsaponifiable matter. The former of these, on chromatographic adsorption on a Tswett column of alumina, using a modified technique in which the separation is conducted in an atmosphere of CO_2, gave a series of coloured zones which on elution and subsequent purification furnished beautifully crystalline specimens of α- and β-carotenes and carotenoids having the spectroscopic and other characteristics of γ-carotene and lycopene.

The physical properties of the specimens of α- and β-carotene so obtained indicate that these are almost certainly purer than any which have previously been described in the literature. The dextrorotation of α-carotene in benzene for the sodium D line is recorded for the first time. Curiously enough, nearly all previous measurements have been made with respect to the less intense cadmium line, for which the value $[\alpha]_{6435} = +385°$ has been generally accepted. The value for the shorter wave-length of the sodium D line corresponding to this cannot be deduced in the absence of an accurate knowledge of the rotatory dispersion of the

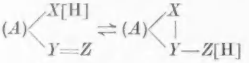
compound, but the rotation of +584° for the helium line 5876, recorded by Smith & Milner [1934] for a specimen melting some degrees lower than ours, is reasonably close to our determination of +640° for the sodium line 5893. Solutions of our specimens of α- and β-carotene in arachis oil which were submitted to Dr H. Wilkinson of our Nutrition Laboratory for biological test provided striking confirmation of Kuhn & Brockmann's [1933, 2] 1:2 ratio for the potencies of α- and β-carotene.

The lycopene zone of the chromatogram of the petrol-soluble unsaponifiable matter, on being eluted and rechromatographed on alumina, furnished upper and lower dark red zones containing lycopene and *neo*lycopene respectively. The closeness of the absorption maxima of *neo*lycopene and γ-carotene would at first sight appear confusing, were it not for the finer differences in adsorptive power which permit the separation of the carotene from the 'total lycopene' zone.

The γ-carotene zone of the original chromatogram, on elution and readsorption on alumina, furnished a single main dark red zone containing γ-carotene. A lighter coloured fringe appeared below this on the second chromatogram, which gave a small quantity of an unidentified carotenoid having maxima of a considerably shorter wave-length than those of γ-carotene. In a subsequent experiment in which more thorough development of the chromatogram of the petrol-soluble unsaponifiable matter was obtained, the zone containing this new carotenoid appeared between γ- and β-carotene. What appears to be *neo*-γ-carotene was obtained when a chromatogram from a concentrate of γ-carotene was very thoroughly developed with a mixture of benzene and light petroleum.

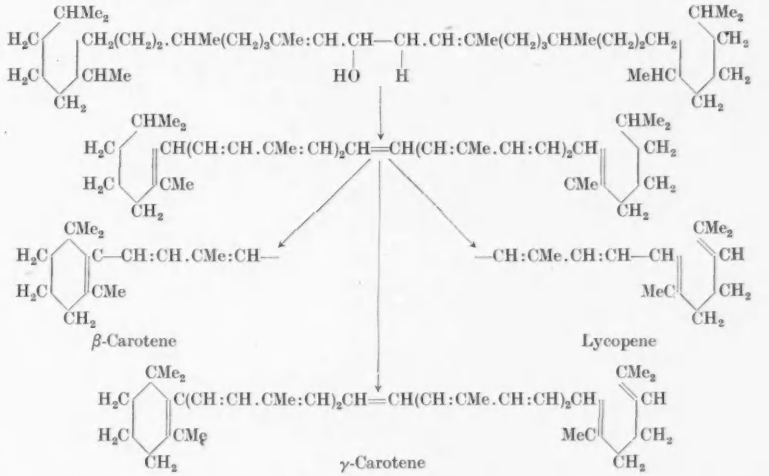
Evaporation of the percolate from the original chromatogram of the petrol-soluble unsaponifiable matter gave a small amount of hentriacontane, accompanied by a quantity of a high-boiling point yellow oil which has not been identified. It may be noted that Carter *et al.* [1939] observed the presence of what appears to be a similar material, which was assumed to be of a terpene character, in algae. The ether-soluble unsaponifiable matter on being dissolved in petrol and partitioned with 90% methyl alcohol gave a hypophasic fraction which on chromatographic adsorption on alumina gave a coloured zone containing *neo*lutein. The epiphasic fraction, which contained quantities of sterols, gave on chromatographic analysis three zones containing substances which showed vague but intense spectroscopic absorption. They gave the usual reaction with chloroformic antimony trichloride with sharply defined maxima in the 590 mμ region and were most probably oxidized carotenoids.

The presence of γ-carotene and lycopene in addition to α- and β-carotenes in this palm oil seems suggestive from the point of view of the biogenesis of these pigments. The photosynthesis of lycopene by dehydrogenation of the product of the benzoin condensation of two molecules of the aldehyde corresponding to phytol has already been suggested by Karrer *et al.* [1930]. That lycopene is the precursor of β-carotene seems to us, however, unlikely, since a ring-chain isomerization of this type finds no parallel in synthetic organic chemistry. If ring-chain prototropy were possible, the presence of the terminal *gem*-dimethyl group of lycopene would, on the basis of the modified strain theory of Ingold and Thorpe, be expected to promote five- rather than six-membered ring formation [cf. Ingold, 1921]. Application of the general expression for ring-chain prototropy

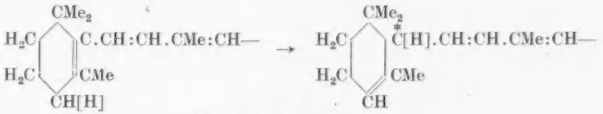


to lycopene would lead to a cyclic isomeride quite different from β-carotene.

It seems more likely therefore that lycopene, β-carotene and also γ-carotene are formed simultaneously from the same intermediate complex, the γ-isomer representing the cyclization of only one end of the hydrocarbon chain:

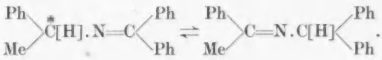


The appearance of α-carotene may be explained as being due to asymmetric isomerization of part of the β-carotene. Such a change would be of the true three-carbon type:

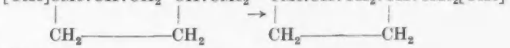


[H] = mobile hydrogen atom.

analogous to the racemization of *d*-benzhydrylidene-α-phenylethylamine [Hsu *et al.* 1935]:



The latter change is concerned with the disappearance of optical activity due to mobility of the azomethine triad system [H]C.N:C⇌C:N.C[H], which is closely related to the three-carbon system [Ingold & Shoppee, 1929]. The corresponding process of asymmetric synthesis evidently occurs in biochemical reactions in view of the frequent appearance of optical activity in natural products. A case of isomeric change involving movement of the centre of asymmetry without loss of optical activity, which is of some interest in this connexion, is furnished by the conversion of linalyl acetate into the correspondingly optically active α-terpinyl ester [Stefan, 1898; Zeitschel, 1906; Ingold & Wilson, 1933]:



The isomeric change in this case is, however, of the anionotropic type and differs from the examples studied by Hsu *et al.* [1935] in that the migrating centre of asymmetry does not follow the route of the mobile group.

Experimental

The sample of 'Sherbro' palm oil used for the following experiments was of recent importation and of good colour. The free fatty acid content determined by titration was 15 %, calculated as palmitic acid.

The oil (1500 g.) was saponified in two batches as follows. A solution of KOH (225 g.) in alcohol (95 %; 1300 ml.) was cooled to 35° and the palm oil (750 g.) was gradually added with shaking. After a short time sufficient soap was formed to render the liquids miscible and light petroleum (750 ml.) and water (400 ml.) were added to the mixture, which was heated to boiling and thereafter kept overnight at laboratory temperature. The mixture was heated under reflux for 1 hr. (*ca.* 45°), cooled, diluted with alcohol (95 %; 3500 ml.) and then with water to produce a final dilution to 15 l. The resulting soap solution was thrice extracted with light petroleum and the united extracts were washed with water until free from soap and alkali and dried over anhydrous Na_2SO_4. Evaporation under reduced pressure in an atmosphere of CO_2 in an all-glass Quickfit apparatus furnished the petrol-soluble unsaponifiable matter which amounted to 5·3 g. This procedure, which was used in all evaporations to dryness described in this paper, enables the temperature to be kept below 50°.

The residual soap solution was further diluted and thrice extracted with methylated ether, the combined extracts being washed, dried and evaporated in CO_2 as in the previous case. The ether-soluble unsaponifiable matter weighed 4·1 g.

The petrol-soluble unsaponifiable matter was resaponified by heating with a mixture of light petroleum (330 ml.), alcohol (670 ml.), benzene (20 ml.) and NaOH (10 g.) for 4 hr. The cooled mixture was twice extracted with light petroleum and the united extracts were washed, dried and evaporated as before. The residue so obtained was dissolved in light petroleum (*ca.* 400 ml.) containing benzene (10 ml.), and the solution was passed down a Tswett column (18 mm. bore) of active alumina (British Drug Houses Ltd.) under slight CO_2 pressure. The chromatogram was developed with a 10 % solution of benzene in light petroleum when the following zones were obtained in order of decreasing adsorption: dark red brown (medium depth, zone 1), dark red (narrow, zone 2), dark orange red (narrow, zone 3), red orange (broad, zone 4) and very pale yellow (medium depth, zone 5). Each zone was separated and the organic material was eluted by alternate washings with acetone and ether and recovered by evaporation under reduced CO_2 pressure.

Zone 1. The material extracted from this zone amounted to 1·56 g.; a portion of it dissolved in alcohol (95 %) furnished a small quantity of a crystalline digitonide. This confirmed the expected presence of sterol, but the material was not further investigated. An attempt to crystallize the original material from light petroleum-methanol gave a sticky residue which showed broad but vague maxima at 480–485 mμ and 455 mμ in carbon disulphide. It gave the usual reaction with chloroformic antimony trichloride with a maximum at 590 mμ.

Zone 2. The extract from this zone furnished 0·28 g. of a somewhat sticky solid which was dissolved in a small amount of benzene. This solution was diluted with light petroleum to give a mixture containing approximately 10 % of benzene and adsorbed on a column of active alumina (British Drug Houses Ltd.).

The chromatogram was developed with a similar mixture of benzene and light petroleum when four zones were obtained below a little oxidized material: dark red (broad, zone 2*a*), very dark red (narrow, zone 2*b*), extremely dark red (medium depth, zone 2*c*) and very dark red (narrow, zone 2*d*).

Zone 2a (*lycopene*). The material extracted from this zone weighed 0·15 g. and showed definite maxima at 544 and 505 mμ and a vague maximum at 481 mμ in carbon disulphide. It gave a reaction with antimony trichloride with a maximum at 582 mμ. From a benzene-methanol solution of this a few mg. of a red solid were obtained (M.P. *ca.* 147°) which showed maxima at 545, 507 and 474–475 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. It appears likely therefore that the displacement of the maximum to 582 mμ in the antimony trichloride reaction with the crude material was due to some colourless impurity which does not affect the maxima of the direct colour spectrum.

Zone 2b (*lycopene, traces of* neo*lycopene*). The residue (0·05 g.) obtained from this could not be crystallized and showed maxima at 543–544, 505 and 478 mμ (vague). It gave a broad maximum at 595 mμ in the antimony trichloride reaction.

Zone 2c (neo*lycopene*). Evaporation of the extract furnished a red gum without any trace of crystalline material which showed maxima at 535, 496 and 469 mμ (vague) in carbon disulphide. This material gave a broad maximum at 595 mμ in the antimony trichloride reaction.

Zone 2d (*γ-carotene*). Evaporation of the extract from this zone gave 0·015 g. of material. No crystals were obtained but portions of the residue showed maxima at 530, 495 and 466 mμ (vague) in carbon disulphide. The antimony trichloride reaction gave a broad maximum at 590 mμ.

Zone 3 (*γ-carotene*). This zone furnished 0·2 g. of a somewhat sticky solid which was dissolved in a small amount of benzene; the solution was diluted with light petroleum and subjected to chromatographic separation on alumina. The chromatogram was developed with a 10 % solution of benzene in light petroleum and formed a single main zone with a lighter-coloured fringe at the bottom. Elution of the main zone gave gummy material which showed bands at 529, 496 and 463 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. A solution of this in benzene-methanol gave a trace of solid which showed maxima at 530, 496 and 463 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. Kuhn & Brockmann [1933, 2] record 533·5 mμ for the first maximum of γ-carotene in carbon disulphide, but Carter *et al.* [1939] have drawn attention to the fact that the first maximum observed in a prism spectrophotometer tends to be situated some few mμ further towards the shorter wave-length region than that seen with grating instruments.

Elution of the lower yellow fringe of the chromatogram gave a trace of material which showed bands at 486, 458 and 426 mμ in carbon disulphide and a maximum at 595 mμ in the antimony trichloride reaction. In a later experiment with the same 'Sherbro' palm oil, this substance was observed between the γ-carotene and β-carotene zones in the large chromatogram of the petrol-soluble unsaponifiable matter. In this experiment a γ-carotene concentrate was obtained which was separated by chromatographic analysis into γ-carotene (maxima at 529, 495 and 465 mμ in carbon disulphide and at 502, 473–474 and 449 mμ in chloroform) and what we believe to be *neo*-γ-carotene (maxima at 520, 488 and 455 mμ in carbon disulphide).

Zone 4 (*α- and β-carotenes*). The mixture of carotenes obtained by elution of

this large zone was dissolved in benzene (100 ml.). An aliquot portion of this diluted with chloroform gave an $E\,1\,\frac{\%}{1\,cm.}$ value of 1710 at 458–459 mμ, indicating a carotene content of about 75 % in the crude material. An alcoholic solution of the latter failed to give a digitonide, suggesting that the colourless impurity was not a sterol.

Portions of the benzene solution corresponding to approximately 0·15 g. of carotene were diluted with light petroleum (9 vol.) and the solutions were passed down Tswett columns of Savory and Moore's 'Mayfair' alumina. The chromatograms were developed with a 25–30 % solution of benzene in light petroleum and the lighter-coloured α-carotene zones were separated from the more strongly adsorbed β-isomer. The pigments were eluted with acetone and the crude carotenes were recovered in the usual way.

A solution of the β-carotene concentrate in acetone deposited a crystalline solid which was collected, dissolved in a 20 % solution of benzene in light petroleum and subjected to chromatographic separation on 'Mayfair' alumina for a second time. The central portion of the β-carotene zone was separated and eluted, and the recovered pigment was crystallized as rapidly as possible from acetone to prevent any isomerization. The β-carotene thus obtained formed beautiful glistening purple red plates, M.P. 184·5° (uncorr.; open melting point tube). A solution of the crystals in chloroform had an $E\,1\,\frac{\%}{1\,cm.}$ value of 2310 at 463 mμ, which is appreciably higher than the value recorded by Gillam [1935] for β-carotene in this solvent. The antimony trichloride reaction showed a maximum at 592 mμ with an $E\,1\,\frac{\%}{1\,cm.}$ value of 423 at the peak of the intensity. It has been observed that the purest specimens of β-carotene which we have obtained in this and other investigations give a rapidly fading maximum in the blue solution obtained in the antimony trichloride reaction.

A solution of the α-carotene concentrate in acetone furnished a crystalline solid which was dissolved in benzene and the solution was diluted with light petroleum and passed down a column of 'Mayfair' alumina. The chromatogram was developed with 30 % benzene in light petroleum and the main zone of α-carotene was eluted. The α-carotene recovered in the usual way was crystallized from acetone within 1½ hr., when it was obtained in glistening red-brown crystals, M.P. 187–187·5° (uncorr.; open melting point tube). This specimen showed maxima at 511, 479 and 450 mμ in carbon disulphide, at 489, 458 and 430 mμ in chloroform, and at 489, 458 and 431 mμ in benzene. The antimony trichloride reaction showed a main band at 495 mμ, a weak band at 650 mμ, an inflexion at 527 mμ and a weak inflexion at 605 mμ. The $E\,1\,\frac{\%}{1\,cm.}$ value for the maximum at 458 mμ in chloroform was 2430.

The optical rotation was determined in benzene solution, using a Hilger polarimeter M. 323 and a G.E.C. Osira sodium lamp as a source of monochromatic light. The instrument was adjusted for a half-shadow angle of 4°. Two determinations, adopting in each case the mean of ten readings, gave the following results:

$$[\alpha]_D = \frac{0{\cdot}162 \times 100^\circ}{2 \times 0{\cdot}0127} = +638^\circ,$$

$$[\alpha]_D = \frac{0{\cdot}276 \times 100^\circ}{2 \times 0{\cdot}02118} = +651^\circ.$$

A similar G.E.C. Osira cadmium lamp gave totally inadequate illumination for use with any carotene solution of sufficient concentration to possess detectable rotation.

Zone 5. Elution of this gave a yellow oil (1·6 g.). A solution of this in alcohol (95 %) gave no precipitate with digitonin. An acetone-light petroleum

solution of the oil furnished a small quantity of hentriacontane, which separated from acetone as a waxy white solid, M.P. 64–65°.

The ether-soluble unsaponifiable matter from the original saponification was dissolved in light petroleum (250 ml.) and the solution was extracted ten times with 90 % methyl alcohol (total of 500 ml.). The petrol solution was dried over anhydrous Na_2SO_4 and evaporated to dryness at a temperature below 50°; the residue was adsorbed on a column of 'Birlec' alumina. The chromatogram developed by a 15 % solution of benzene in light petroleum formed three zones: dark red (broad; zone 6), orange red (medium depth; zone 7) and yellow ochre (narrow; zone 8).

Zone 6. An acetone extract from this furnished crystalline sterols and an alcoholic solution of these gave a copious precipitate of digitonides, neither of which were investigated. The colouring matter present showed a broad and vague maximum at about 485–490 mμ in carbon disulphide and gave the usual reaction with chloroformic antimony trichloride with a maximum at 590 mμ.

Zone 7. This furnished a gummy material which showed a similar vague maximum in the 485 mμ region and gave a band at 588 mμ in the antimony trichloride reaction.

Zone 8. This also failed to furnish a crystalline pigment and gave a gum which showed maxima at 505 and 480 mμ in carbon disulphide. The antimony trichloride reaction showed a transient band at 590 mμ, a weak but persistent band at 495 mμ and a slight maximum at 650 mμ which increased during observation.

The hypophasic portion of the ether-soluble unsaponifiable matter recovered from 90 % methyl alcohol was subjected to chromatographic adsorption on a column of 'Birlec' alumina. The chromatogram, although developed with a 35 % solution of benzene in light petroleum, furnished only a single orange red zone (medium depth; zone 9).

Zone 9 (neo*lutein*). The crude carotenoid obtained from this zone formed a gum which gave a negative reaction with alcoholic digitonin. It showed maxima at 501 and 473 mμ in carbon disulphide and a band at 595 mμ in the antimony trichloride reaction.

Summary

1. Examination of the unsaponifiable matter of 'Sherbro' palm oil revealed the presence of α-, β- and γ-carotenes, accompanied by lycopene, *neo*lycopene, *neo*lutein and what appears to be *neo*-γ-carotene. A new carotenoid which is adsorbed at a position intermediate between γ- and β-carotenes on the chromatogram was also observed.

2. The physical properties of the specimens of α- and β-carotenes so obtained indicate that these are probably somewhat purer than any which have been described in the literature.

3. The biogenesis of carotenoids is discussed in the light of the phenomena of ring-chain tautomerism and prototropic change involving asymmetric isomerization. It is suggested that lycopene, β-carotene and also γ-carotene are formed concurrently from the same intermediate complex and that α-carotene may arise from subsequent isomerization of the β-compound.

We wish to thank the directors of Lever Brothers and Unilever Ltd. for permission to publish these results. We also wish to express our thanks to Dr J. R. Edisbury for his help in connexion with some of the spectroscopic measurements.

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6. THE ANALYSIS OF BLOOD PHOSPHOLIPINS

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While the importance of phospholipins in the metabolism of fats is universally recognized, the most perfunctory examination of the relevant literature is sufficient to show that the methods of analysis at present available are inadequate. Though it is possible to determine with ease and accuracy the total phospholipin content of any biological specimen, an estimate of the partition of the lipoid P among the various phospholipins is by no means as simple. Few workers have even attempted such analyses, and none of the methods proposed is completely satisfactory. Thannhauser *et al.* [1939] have determined sphingomyelin by precipitation of the whole molecule with ammonium reineckate by means of a technique published in an earlier series of papers from the same school. Hydrolysis of the lipin solution with baryta is the preliminary step in their choline estimation, and the concentration of lecithin is estimated from the difference between total choline and total lipoid P. The principal drawback to this method is the amount of material required if the sphingomyelin precipitation is to be really satisfactory. On a small scale it seems that precipitation is frequently incomplete.

A method requiring only very small amounts of material has been published by Kirk [1938]. It is based fundamentally on the difference in the solubilities of the various phospholipins in different solvents. While this principle is perfectly satisfactory as long as its application is restricted to qualitative work on a large scale, it breaks down when applied to the quantitative analysis of phospholipins amounting altogether to hardly more than a milligram. This, however, is apparently not the only fault in Kirk's technique, because his total phospholipin values are far more variable than those generally accepted, and many of them are exceedingly low. In one case, for example, he reports a total phospholipin concentration in red blood corpuscles of 26 mg. per 100 ml. This corresponds to a lipoid P value of about 1 mg. per 100 ml., whereas the normal limits of 10–14 mg. per 100 ml. have rarely been passed in either direction by other analysts. Finally, it may be remarked that Kirk appears to have found little difference between the concentration of phospholipins in erythrocytes and in plasma, whereas it is well known that it is generally greater in the former.

Even before the appearance of these papers we had been engaged on this problem for some time, and it had seemed to us that it was vain to hope for a solution based on solubility differences, especially when working with the small samples which we were proposing to use. Our attention, therefore, was directed towards the possibility of determining the various characteristic residues of phospholipin molecules, such as choline, glycerol and β-aminoethanol, with a view to calculating from them the concentrations of the phospholipins. It is readily seen from Table 1 that the calculation of lecithin, kephalin and sphingomyelin is possible from choline, glycerol and P values, and that β-aminoethanol determinations would afford a very useful check on the other analyses.

Table 1

Substance	Phosphorus	Choline	Glycerol	Colamine
Lecithin	1	1	1	—
Kephalin	1	—	1	1
Sphingomyelin	1	1	—	1

In this paper are described methods for the determination of choline and glycerol, and their application, as indicated above, to specimens of phospholipins prepared from 2 ml. blood: that is to say, about 5–7 mg. mixed phospholipins. Mention is also made of hitherto unsuccessful attempts to evolve a specific micro-method for the determination of β-aminoethanol, and hence of kephalin.

The determination of choline

Of the reagents generally used nowadays for the precipitation of choline we have preferred ammonium reineckate to iodine because the precipitate of choline reineckate is much easier to work with than that of choline enneaiodide. The analysis of the precipitate is also much simpler, although it must be admitted that the application of the reineckate method is limited by the fact that choline reineckate is somewhat more soluble than the enneaiodide.

The method described here is a modification of that of Beattie [1936], whose technique we have found satisfactory except in a few minor details. Our method, however, is very much more sensitive than Beattie's, and can be applied to 1 ml. of a solution containing as little as 0·25 μmol. (0·03 mg.) choline.

Method

Reagents. Barium hydroxide, saturated solution.

H_2SO_4, approximately N and $N/10$.

Ammonium reineckate.

Acetone, ammonia-free, prepared by the distillation of A.R. acetone once from slightly acid solution.

Digestion mixture [Folin & Wu, 1919] diluted with 3 vol. of water.

H_2SO_4, 0·007 N, prepared with indicator according to Conway [1939].

NaOH, 40 % and 0·007 N.

Procedure. A volume of lipin solution containing not more than 4·5 μmol. (0·54 mg.) of choline is evaporated to a small volume in a 25 ml. pyrex conical flask with 1 ml. saturated aqueous baryta and the mixture is heated on the steam bath for 1 hr. A further 1 ml. of baryta is then added and the heating is continued for 1 hr. more. The mixture is neutralized to methyl red with H_2SO_4, using first N and then, as the end-point is approached, $N/10$ acid. The solution, with the suspended precipitate of barium sulphate and fatty acids, is evaporated to 0·3–0·5 ml. and transferred to a small filter paper with the aid of a capillary pipette and a rubber teat. The precipitate is washed with successive amounts of hot water until the total volume of filtrate amounts to not more than 1·5 ml. On no account whatever must the filtrate be alkaline, and it should not be more acid than $N/50$. 1 ml. of a cold freshly-prepared saturated aqueous solution of ammonium reineckate is then added and the tube is left in the refrigerator for 20–30 min. to ensure complete precipitation. The pale pink crystalline precipitate of choline reineckate is filtered off through a tiny pad of cotton-wool pressed into the bottom of a small filter-funnel (paper is very difficult to wash free from excess ammonium reineckate) and washed with ice-cold water and a little ether. It is dissolved by pouring a few drops of ammonia-free acetone through the

filter, the resulting pink solution being collected in a pyrex test tube. After the addition of a drop of dilute acid to prevent loss of ammonia during the subsequent heating, the acetone is removed by cautious evaporation over a free flame. 0·5 ml. of dilute digestion mixture is then added and the precipitate is ashed. The digest is made strongly alkaline and distilled with steam into 5 ml. 0·007 *N* H_2SO_4. The excess acid is titrated with 0·007 *N* NaOH. The formula of choline reineckate is $HO.C_2H_4.N(CH_3)_3.[Cr(NH_3)_2(CNS)_4]$ and from this it can be seen that 1 mol. of choline yields ultimately 7 mol. of ammonia. Consequently, each ml. 0·007 *N* alkali corresponds to 1 μmol. choline. Table 2 shows that very satisfactory duplicate analyses can be made and that the recovery of added choline is, within the limits of experimental error, quantitative. It is theoretically necessary to do a 'blank' experiment on unhydrolysed lipin extract, but in the case of whole blood we have found the 'blank' to be so very small as to be negligible. This, of course, does not apply to the ammonia-blank of the Kjeldahl determination.

Table 2. *Choline, millimol./100 ml. blood*

Sample	Choline added	Choline found	Recovered	
			Amount	%
1	—	0·256, 0·257	—	—
2	—	0·217, 0·223	—	—
3	—	0·267, 0·262	—	—
4	—	0·217, 0·217	—	—
5	—	0·268, 0·275	—	—
6	—	0·220, 0·226	—	—
7	—	0·273, 0·264	—	—
8	—	0·216, 0·218	—	—
8	0·215	0·437	0·220	102
8	0·215	0·429	0·212	99

It remains to discuss the specificity of the method. Ammonium reineckate appears to be a highly specific precipitant for quaternary bases. It has been used for the isolation not only of choline and acetylcholine, but also of neurine, carnitine and sphingomyelin. Lecithin does not, on the other hand, appear to form an insoluble reineckate, and creatinine does not seem to be precipitated until its concentration reaches the quite unphysiologically high level of 5 mg. per ml. Hence, while it is in theory possible that blood might contain appreciable amounts of reineckate-precipitable bases, 'blank' estimations have shown that the amount present is not significant, and in any case it is difficult to imagine any compounds other than the choline-containing phospholipins (and, of course, acetylcholine, which has been shown by Bischoff *et al.* [1931] to be present only in very low concentration) from which reineckate-precipitable base would be liberated by hydrolysis with baryta. Furthermore, we have prepared by the method outlined above a specimen of reineckate from 600 ml. of alcohol-ether extract of blood (equivalent to 22 ml. blood) which, when analysed for nitrogen, gave a value of 23·3 %. The theoretical value for choline reineckate is 23·2 %, but this close agreement does little more than confirm the absence of gross contamination, since six-sevenths of the total nitrogen comes from the reineckate radicle. The crystalline form of the precipitate, however, seemed identical with that of the reineckate prepared from pure choline chloride solution, and it would appear that there might be no great danger in the assumption that choline is actually precipitated as a fairly pure reineckate in these experiments.

The determination of glycerol

The determination of glycerol has never been a simple procedure. On an industrial scale the purity of glycerol is sometimes checked by means of a quantitative chromic acid oxidation. Such a technique is obviously quite unsuitable for the present purpose. More hope is offered by permanganate oxidation. Alkaline permanganate oxidizes glycerol with fair regularity to oxalate, which can then be determined in the usual way by precipitation as the calcium salt. This method has been applied to lipoid glycerol [Dickson, 1935], but has been shown to give somewhat erratic results, largely because of the difficulty of determining oxalate accurately in very low concentration. Fleury & Fatome [1935] have recently published a method which depends on the oxidation of glycerol with an acid solution of sodium or potassium periodate and oxidimetric determination of the excess periodate, in accordance with the following equation:

$$CH_2OH.CHOH.CH_2OH + 2HIO_4 \rightarrow 2HIO_3 + 2HCHO + HCOOH + H_2O.$$

The method as published proved unsuitable for two main reasons: in the first place, it deals with amounts of glycerol which are far too large (5–15 mg.), and in the second, it simply measures the disappearance of an oxidizing agent whose specificity is by no means high. It appeared to us, however, that a solution to the problem might be found in the determination of formaldehyde, since each molecule of glycerol yields two molecules of that compound in this oxidation. While many substances are attacked by periodic acid, comparatively few of these (in general, only primary alcohols in which the primary alcoholic group is directly linked to another partially oxidized carbon atom) yield formaldehyde. The number of these which would be liable to interfere with the present experiments is limited, and the most likely one, glucose, can be readily removed by treatment with copper sulphate and calcium hydroxide. Glycerophosphates would also presumably interfere, but these have been shown by Stewart & Hendry [1935] not to be extracted from blood by the usual alcohol-ether mixture.

Formaldehyde, however, is by no means easy to estimate accurately on a micro-scale, although Schryver [1910] has described an exceedingly delicate and highly specific colour reaction for its detection in minute concentration. Schryver himself claimed that his own technique was satisfactory for quantitative use, but in our hands it has given inconsistent and inaccurate results, owing apparently to side reactions among the various reagents which Schryver employed in rather high concentration. We have consequently found it necessary to modify his technique very considerably, and the glycerol method incorporating the modifications is described below.

Reagents. NaOH, 20 % solution.

Copper sulphate cryst., A.R., 10 % solution.

Calcium hydroxide, as pure as possible.

Sodium periodate, 0·05 *M* in 3 *N* H_2SO_4 (formula of salt supplied by B.D.H. $Na_3H(IO_5)_2$).

Sodium thiosulphate, 50 % solution.

Phenylhydrazine hydrochloride, 'Analar', 0·03 *M* in 0·2 *N* HCl.

Potassium ferricyanide, 'Analar', 0·015 *M*. Neither of these last two solutions should be more than a week old, and both should be kept in the dark.

H_2SO_4, 20 % by volume.

Procedure. To a volume of lipin solution expected to contain not less than 0·2 μmol. (about 0·02 mg.) glycerol is added 0·2 ml. 20 % NaOH. The solution is

made up to about 10 ml. with alcohol and evaporated on the hot-plate until its volume does not exceed 1 ml. This evaporation should take about 45 min., and great care must be taken that at no time does any part of the bottom of the flask become dry, otherwise appreciable quantities of glycerol will be volatilized and lost. The residual fluid is transferred with the aid of a capillary pipette to a narrow centrifuge tube graduated at 3 ml., and the flask is rinsed out with several small volumes of hot water. When the solution has cooled to room temperature, 0·3 ml. 10 % copper sulphate, a knife-point of slaked lime and sufficient water to make up the volume to 3 ml. are added. The tube is left to stand for 1 hr., during which it is frequently shaken, and it is then centrifuged. An aliquot of the supernatant fluid, usually 2·5 ml., is pipetted into a 10 ml. round-bottomed pyrex flask with a neck ground to fit a micro-Kjeldahl head with the long tube removed (see Fig. 1). 1 ml. acid sodium periodate solution is added and the flask left to stand for 4–5 min. (not longer). The excess periodate is reduced to iodide by the dropwise addition of 50 % sodium thiosulphate,[5] of which excess should be avoided. The contents of the flask, after neutralization with 20 % NaOH (3 or 4 drops extra being added), are distilled into 2 ml. acid phenylhydrazine solution. When the flask is almost dry a little water is added and the distillation is repeated to ensure that all the formaldehyde passes over. It is important that the solution should not become acid during the distillation. Methyl red and phenol red are suitable indicators for observing this precaution. The distillate is diluted to about 20 ml. and 2 ml. potassium ferricyanide are added. After 25–30 min., during which a colour varying from pale yellow to a rich dark sherry develops, the solution is diluted in a 50 ml. volumetric flask to about 30 ml., and 10 ml. 20 % H_2SO_4 are added. This changes the colour to a rose-red. The solution is made up to the mark with water and compared with a standard made by oxidizing a measured volume of standard glycerol solution. Since the intensity of the final pink colour is not quite proportional to the concentration of formaldehyde, and hence to the concentration of glycerol, it is important to employ a standard the concentration of which approaches that of the unknown fairly closely. Table 3 gives a few typical results, including not

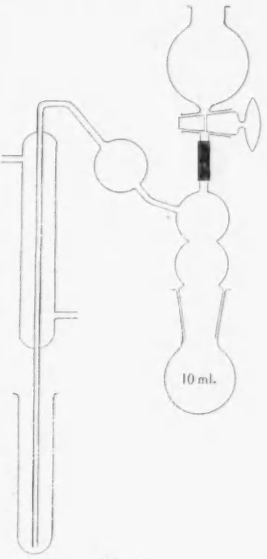

Fig. 1.

Table 3. *Glycerol, millimol./100 ml. blood*

Sample	Added	Found	Recovered	
			Amount	%
1	—	0·088, 0·090	—	—
2	—	0·127, 0·124	—	—
3	—	0·076, 0·0735	—	—
4	—	0·152, 0·147	—	—
5	—	0·178, 0·176	—	—
1	0·1025	0·197	0·108	105
2	0·1025	0·221	0·096	94
2	0·1025	0·224	0·099	97

only duplicate analyses of alcohol-ether extracts of whole blood but also recoveries of added glycerol.

These two methods have been applied, in conjunction with a standard lipoid P technique [Stewart & Hendry, 1935], to specimens of phospholipins prepared from whole blood in the following way.

50 ml. alcohol-ether extract of blood [Bloor, 1928] were evaporated just to dryness on the hot-plate and finally in the steam-oven. The residue was immediately taken up in light petroleum and the turbid solution transferred to a centrifuge tube where it was concentrated to about 0·3 ml. by immersion in a beaker of hot water. Acetone was then added from a 5 ml. pipette, very slowly and with continuous shaking so that the phospholipins separated as a flocculent white precipitate and not as a tarry mass. Precipitation was completed by adding 3 drops of a saturated alcoholic solution of magnesium chloride and allowing the tube to stand for 2 hr., preferably in the refrigerator. At the end of that time any precipitate adhering to the walls of the centrifuge tube was carefully removed by scratching and the precipitate was thrown down in the centrifuge. It invariably settled rapidly and completely and was twice washed at the centrifuge with 5 ml. cold acetone before being taken up in 10 ml. of a mixture of 3 parts of ether, 2 of alcohol and 1 of water, in which it gave a perfectly clear, almost colourless solution. The entire procedure could be carried out in about 3 hr., and during this time the phospholipins spent 2 hr. in the refrigerator, which should have minimized the oxidation by atmospheric oxygen to which these compounds are so very susceptible. Analyses showed that the procedure extracted 92–97 % of the P in the alcohol-ether extract, so that not only is this P virtually all phospholipin-P, but the extraction and precipitation with acetone and alcoholic magnesium chloride are also satisfactory and complete.

Aliquot portions of the ether-alcohol-water solution were analysed for glycerol (2 ml.) and choline (2 ml.) by the methods just described, and for phosphorus (1 ml.) by the method of Stewart & Hendry [1935]. The results of 12 analyses on specimens of whole blood from healthy men in their early twenties are given in Table 4. Table 5 shows the concentrations of lecithin, kephalin and sphingomyelin calculated from the figures in Table 4, the calculations being based on the assumption that these are the only choline-, phosphorus- and glycerol-containing compounds present in the material analysed; this Table also shows the same results calculated as percentages of the total phospholipins.

The figures in Table 5 show one or two rather interesting facts. In the first place, it is clear that although the phospholipins of blood have often been loosely described as 'lecithin', lecithin itself actually accounts for only a small proportion

Table 4

No. of sample	Phosphorus milliatoms/100 ml. blood	Choline millimol./100 ml. blood	Glycerol millimol./100 ml. blood
1	0·432, 0·420	0·274, 0·283	0·178, 0·176
2	0·333	0·290, 0·298	0·147, 0·151
3	0·355	0·300	0·109
4	0·336	0·283	0·124
5	0·340	0·255	0·119
6	0·366	0·271	0·138
7	0·316	0·256	0·111
8	0·319	0·227	0·127
9	0·352	0·275	0·108
10	0·351	0·245	0·104
11	0·347	0·261	0·103
12	0·380	0·292	0·113

Table 5. *Concentrations of phospholipins in blood samples*

No. of sample	Lecithin		Kephalin		Sphingomyelin	
	millimol./ 100 ml.	% of total	millimol./ 100 ml.	% of total	millimol./ 100 ml.	% of total
1	0·028	6	0·148	35	0·250	59
2	0·110	33	0·039	11	0·184	56
3	0·054	15·5	0·055	15·5	0·246	69
4	0·071	23	0·053	14	0·212	63
5	0·034	13	0·085	25	0·211	62
6	0·043	11	0·095	26	0·228	63
7	0·052	16	0·059	19	0·204	65
8	0·035	14	0·092	26	0·192	60
9	0·031	9	0·077	22	0·244	69
10	0·000	0	0·104	30	0·245	70
11	0·017	5	0·086	25	0·244	70
12	0·025	9·5	0·078	20·5	0·267	70
	Mean	13		22		64

of the total. In one case (No. 10), indeed, no lecithin at all was found. While errors in estimations will have been magnified in the calculations, it seems highly improbable that this low concentration of lecithin would be either artificial or fortuitous in every one of 12 consecutive analyses. A second point of interest in these tables is that sphingomyelin is quantitatively by far the most important of the phospholipins present. Thirdly, in nearly all cases the concentration of kephalin is considerably greater than that of lecithin. Even so short a series, moreover, has demonstrated clearly that the proportion of sphingomyelin is fairly constant. It seems possible that this constancy might be emphasized by a series of analyses on plasma and corpuscles separately.

It is difficult to compare these findings with those of Kirk [1938], since, as we have already mentioned, there appear to be grounds for doubting the accuracy of his results. Other workers have, however, recently published comparable figures. Williams *et al.* [1938] have given figures for kephalin concentrations in blood plasma and corpuscles obtained in much the same way as our own by difference between lipoid P and choline. Their results are somewhat higher than we should have expected from our own analyses of whole blood, but this may be explained by the fact that they carried out the precipitation of choline reineckate in a solution sufficiently acid to interfere with the precipitation. This may have led to low results for choline and consequently high kephalin figures. The latter, nevertheless, are of the same order as our own.

Other interesting figures are those of Thannhauser *et al.* [1939] for blood serum and various tissues. These again show a remarkable constancy in sphingomyelin concentration, although it must be admitted that as a rule their results for serum sphingomyelin are rather lower than we should expect from our results with whole blood.

The phosphorus-fatty acid analyses of Stewart & Hendry [1935] indicated that approximately one-half of the total phospholipins of whole blood consisted of sphingomyelin, a proportion somewhat lower than that observed in the present work. Neither series is long, so that the difference may be a chance one. On the other hand, Stewart & Hendry based their admittedly rough conclusion on the ratio of P atoms to fatty acid molecules in the phospholipin precipitate, and a very slight oxidation of the fatty acids before estimation would result in a shift of this ratio sufficient to account for the observed discrepancy. For a mixture containing 50 % sphingomyelin the ratio P:—COOH is 1:1·5; for a

mixture containing 64 % it is 1:1·36. Hence, a 10 % increase in the number of carboxyl groups is all that it is necessary to postulate in order to make the two sets of figures agree, and it seems not unreasonable to suggest that such a small amount of oxidation may have taken place. A few preliminary analyses have suggested that on occasion the increase of carboxyl groups may be very much greater than this, and this conclusion is supported by the work of Paal [1929] who studied the oxidation of alcoholic solutions of lecithin and found the number of free carboxyl groups to increase very rapidly.

The attempted estimation of β-aminoethanol and kephalin

During the investigation of substances which might have interfered with the determination of glycerol, it was observed that β-aminoethanol was oxidized slowly in acid and rapidly in alkaline solutions of periodate in the following way:

$$CH_2OH.CH_2NH_2 + IO_4^- \rightarrow IO_3^- + 2HCHO + [redacted] + NH_3.$$

This equation has been verified, not only by the estimation of the periodate used up and the formaldehyde produced, but also by the determination of the ammonia. Several methods were used, depending on the quantities of β-aminoethanol under consideration, and much the most satisfactory proved to be the method of Conway [1939], with which it was practicable to determine accurately the ammonia from as little as 0·2 μmol. β-aminoethanol. The reaction, which appeared to be highly specific, was carried out simply by mixing in the outer chamber of a Conway unit 0·5–1·5 ml. β-aminoethanol solution, 0·5 ml. 0·05 *M* sodium periodate (dissolved in water with the minimum amount of H_2SO_4) and 1 ml. saturated aqueous K_2CO_3. The ammonia formed was allowed to diffuse into standard H_2SO_4 in the inner chamber, the excess acid being titrated after the completion of the diffusion. When the method was applied to hydrolysed lipoid extracts figures which were certainly of the right order were obtained, but it did not seem possible, except on rare occasions, to recover more than 65–75 % of added β-aminoethanol. Various methods were tried for the purification and separation of the base before estimation; these did not alter materially the results of the analysis, but they did not enhance the recovery of added β-aminoethanol to any great extent. A certain amount of success, but not sufficient to justify the use of the method, attended the adsorption of the β-aminoethanol, after hydrolysis of the lipins, from slightly alkaline solution on to frankonit KL, followed by periodate oxidation of the base *in situ* on the adsorbent.

Summary

Methods for the determination of choline and glycerol in lipin solutions are described, with their application to the separate determination of the various phospholipins in whole blood.

In 12 blood samples the concentration of sphingomyelin ranged from 56 to 70 % of the total phospholipin, with a mean of 64 %. In most of the cases the concentration of kephalin appeared to be considerably greater than that of lecithin.

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7. THE PLASMA-CELL PARTITION OF BLOOD LEAD

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The distribution of lead between the cells and plasma of blood has been the subject of various reports. According to Aub *et al.* [1926], in experimental lead poisoning 80 % of the lead is in the plasma; Blumberg & Scott [1935] find 90 % in the cells in lead poisoning in man and at least half and usually more in the cells in normal blood; Teisinger [1936] finds that the lead is divided between the cells and plasma in a variable ratio, while Willoughby & Wilkins [1938] could detect no lead in the serum of 90 % of samples of normal blood.

In view of these different findings a further investigation of the plasma-cell partition was undertaken and the results are reported here.

Experimental

For the determination of lead in fresh human blood approximately 40 ml. blood were withdrawn from a vein and mixed with a minimal amount of potassium oxalate Analar; one portion was then centrifuged and the plasma separated. Lead was estimated in the whole blood and plasma by the method described previously [Tompsett & Anderson, 1935; 1939; Tompsett, 1939]. In two cases a determination of cell volume was made by means of the haematocrit and the actual lead concentration in the cells calculated. The results obtained with blood from one normal laboratory worker, two hospital patients with a history of possible exposure to lead but with no obvious poisoning, and three cases of lead poisoning, are given in Table 1. It will be seen that the ratio of whole blood lead to plasma lead is fairly constant, irrespective of the amount of lead present. The concentration in whole fresh blood is approximately three times that in the plasma. Case No. 6 is exceptional in having a ratio of only 1·6 : 1.

A supply of normal stored blood was available in bottles discarded from a blood bank at the end of the period of storage. It was possible to use large volumes of this blood, and 100 ml. of whole blood and 100 ml. of plasma were taken from each bottle for analysis. A correction was made for the dilution due to the sodium citrate and saline anticoagulant solution in the bottles and for the small amount of lead in this solution. The results of the analyses of eight samples, which are given in Table 1, are thus calculated for the original blood, and show a distribution of lead between the cells and plasma similar to that found for fresh blood, but with a slightly higher ratio of lead in whole blood.

Two young male rabbits were given lead by mouth in the form of lead acetate mixed with a diet of crushed oats and bran. They received approximately 30 mg. of lead per kg. per day, the one for 8 and the other for 24 days. Blood films were examined for punctate basophilia at intervals, and at the end of the periods of lead administration the animals were anaesthetized with ether, bled by heart

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Table 1. *Lead concentration in μg. per 100 ml. whole blood, plasma or cells*

Disease	Whole blood	Plasma	Cells	Whole blood / Plasma
1. Normal	69	28	—	2·5
2. Lead poisoning	136	46	—	2·9
3. Lead poisoning	366	118	—	3·1
4. Neuritis	40	15	—	2·7
5. Lead poisoning	128	45	258	2·8
6. Undiagnosed	46	28	73	1·6

Stored normal human blood

Whole blood	Plasma	Whole blood / Plasma
76	27	2·8
24	7	3·4
43	12	3·6
31	9	3·4
48	14	3·4
20	6	3·3
20	5	4·0
58	18	3·2

puncture and killed. The blood was allowed to clot and lead was estimated in the separated serum and clot. Two other young males were each given a single dose of 0·5 g. lead acetate in solution by stomach tube, and blood was taken as before, in the one 20 hr. and in the other 48 hr. after lead administration, and lead was again estimated in the serum and clot. Blood lead was also estimated in a control litter-mate and in two stock female rabbits. The concentration of lead in the whole blood was found by adding the total amount in the serum to the total in the clot and correcting for volume. The results are shown in Table 2. The distribution in normal rabbit blood was of the same order as in blood from human subjects. Rabbits nos. 2 and 3, receiving lead in the food, had a raised blood lead, no. 2, which received lead for 8 days, showing a slight relative increase in the serum lead, while in no. 3, which received lead for 24 days, the serum lead concentration was the same as that of the whole blood. Administration of lead in one large dose by stomach tube caused a greater rise in whole blood lead; in no. 4, 20 hr. after administration of lead, the concentration in the serum was nearly equal to that in the whole blood, while in no. 5 the serum lead concentration was 50 % greater than that of the whole blood.

Table 2. *Rabbit blood: lead concentration in μg. per 100 ml. whole blood or serum*

	Whole blood	Serum	Whole blood / Serum
1. Control	37	17	2·2
2. 30 mg. lead per kg. for 8 days	79	41	1·9
3. 30 mg. lead per kg. for 24 days	85	86	1·0
4. 0·5 g. lead acetate by stomach tube, bled 20 hr. afterwards	102	96	1·0
5. 0·5 g. lead acetate by stomach tube, bled 48 hr. afterwards	112	153	0·7
6. Normal female	11	4	2·75
7. Normal female	53	21	2·5

The blood films of these animals all showed a great increase in polychromatic cells after lead administration, but no. 2 was the only one which had a definite punctate basophilia.

In view of the possibility that lead might be present in the cells as a soluble soap, e.g. lead oleate, a sample of 20 ml. of fresh normal human blood was extracted with alcohol-ether mixture, as in Bloor's method for the extraction of the total lipoids of blood, and then lead was estimated in the extract and residue. No appreciable amount of lead was found in the extract, while the residue contained a normal amount.

Discussion

The accuracy of the analytical method employed is of paramount importance in investigations of this nature, and faults in the methods used may account for some of the widely different distributions of lead in the blood which have been reported. We have criticized some of these methods in a previous paper, in which we first described the method at present in use [Tompsett & Anderson, 1935]. This method consists essentially in the extraction and separation of the lead as an ether-soluble complex with sodium diethyldithiocarbamate and its subsequent estimation colorimetrically with dithizone. Kench [1940] has used this method with slight modifications for the estimation of lead in urine and obtained accurate results. Unfortunately he is wrong in stating: 'Tompsett & Anderson [1935; 1939] have extracted lead from biological materials as a complex either with sodium diethyldithiocarbamate or with dithizone, both substances being stable in the presence of ammoniacal cyanide.' One of the objects of our first paper was to advocate the use of sodium diethyldithiocarbamate instead of dithizone for the extraction of lead. Speaking of the dithizone method we said, 'we have found that in practice, when used to separate lead from solutions of the ash of urine, liver etc., the method gave erratic results'.

The relatively constant ratio between the concentrations of lead in human whole blood and in plasma, which is the same in normal bloods as in those from cases of lead poisoning, suggests that an equilibrium is reached in the distribution between cells and plasma. By contrast, when in the rabbits active absorption of lead was taking place this equilibrium was not reached and lead was present in greater concentration in the plasma. Thus it is possible to reconcile Aub's finding of 80 % of lead in plasma in experimental lead poisoning with the later reports that most of the lead is in the cells in clinical lead poisoning.

The form in which lead is present in the blood is undecided. Most of the investigations of the question have been made on serum or blood to which lead salts have been added *in vitro*, or on the blood after intravenous injection of lead salts. These experiments can all be criticized on the grounds that they are unphysiological. The findings reported here do not provide any data as to the nature of blood lead, but exclude the possibility of an ether-soluble form.

Summary

1. The concentration of lead in human whole blood was approximately three times that in the plasma of normal subjects and of patients with lead poisoning.

2. In stored blood the ratio of lead concentration in whole blood to that in plasma was slightly higher than in fresh blood.

3. In rabbits absorbing lead by mouth the concentration of lead in the plasma was equal to or greater than that in whole blood.

4. The lead in normal human blood was not extracted by ether.

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8. STUDIES IN DETOXICATION

8. ON THE ALLEGED FORMATION OF *p*-HYDROXYLAMINOBENZENESULPHONAMIDE AND *p*-AMINOPHENOL FROM SULPHANILAMIDE *IN VIVO*, ON THE COLOUR REACTIONS USED FOR THEIR DETECTION, AND ON THE POSSIBLE FORMATION OF AMINOPHENOLSULPHONAMIDES

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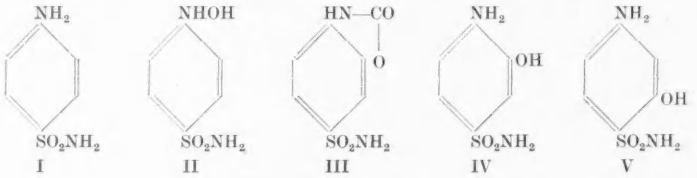
When sulphanilamide is adminstered to an animal, it is mainly excreted free and acetylated; a small portion, however, undergoes oxidation, and since the oxidized portion may be responsible for certain toxic effects, its characterization becomes a question of importance. So far reliable identification of the oxidation products of sulphanilamide has not been accomplished, although a few suggestions as to their nature have been offered [e.g. Mayer, 1937; James, 1940; Rimington & Hemmings, 1939; Rosenthal & Bauer, 1939]. The compounds most favoured have been *p*-hydroxylaminobenzenesulphonamide and *p*-aminophenol; claims that these substances appear in urine after ingestion of sulphanilamide have been based mainly on certain colour reactions. It is one object of this paper to show that these reactions are not specific and do not in any way prove the presence of these substances.

A consideration of what is known about the biological oxidation of various aromatic compounds suggests that oxidation of sulphanilamide (I) *in vivo* might yield the following compounds: *p*-hydroxylaminobenzenesulphonamide (II), 2-oxybenzoxazole-6-sulphonamide (III), 1-amino-2-hydroxybenzene-4-sulphonamide (IV) or 1-amino-3-hydroxybenzene-4-sulphonamide (V).



There is the further possibility that dihydric phenols or 4:4′-disulphonamidoazoxybenzene might be formed. The aminophenols IV and V could be excreted in conjugation, the hydroxyl groups with glucuronic acid or sulphuric acid and the amino groups with acetic acid. It is important to remember this when some of the colour tests are applied.

[1] Working under Caroline Harrold Research Grant, University of Birmingham.

The stability of the sulphonamide group [cf. Gray *et al.* 1937] makes it improbable that it would be eliminated *in vivo*, so that, on theoretical grounds, the formation of *p*-aminophenol from sulphanilamide is unlikely. We have, nevertheless, examined carefully a number of urines, both human and rabbit, but in no case have we detected *p*-aminophenol. The indophenol reaction which has been frequently used for the detection of *p*-aminophenol can be misleading, and we have devised a modification of the test which is less liable to misinterpretation.

Hydroxylamine derivatives have been postulated as intermediates in the metabolisms of aryl-amino and nitro compounds by several workers [e.g. Ellinger, 1920; Lipschitz, 1920; Böhm, 1939] and some claims to the isolation of such derivatives have been put forward. In 1937 Mayer postulated the slow formation of *p*-hydroxylaminobenzenesulphonamide in the body as the basis for the activity of sulphanilamide. This view has received support from workers who claim to have detected this hydroxylamine in urines of man, dogs, rabbits and mice receiving the drug [e.g. James, 1940; Rosenthal & Bauer, 1939; Rosenthal, 1939, 1]. The claims have been largely based on two colour reactions which, as we shall show, are not specific. We venture to suggest that some of those who support the view that *p*-hydroxylaminobenzenesulphonamide is formed and excreted in the biological oxidation of sulphanilamide have been unduly influenced by the analogy that β-phenylhydroxylamine is supposed to be formed during the biological oxidation of aromatic amines such as aniline and acetanilide. Brownlee [1939], Rimington & Hemmings [1939], and James [1940], for example, state that Ellinger [1920] isolated acetylphenylhydroxylamine from the blood of cats poisoned with acetanilide. Now this is not true. Ellinger isolated 1·8 mg. of a substance which he suggested was acetylphenylhydroxylamine 'vorbehaltlich der übereinstimmenden Elementaranalyse'. The analysis was not performed and his other evidence was not conclusive. Again, Lipschitz [1920] claimed that *m*-nitrophenylhydroxylamine was formed from *m*-dinitrobenzene by excised tissues. His evidence consisted of comparative tests unsupported by isolation or any specific test. Undue importance has been given to the power of β-arylhydroxylamines to form methaemoglobin in blood. This is a property shared with several substances, including 1-amino-2-hydroxybenzene-4-sulphonamide [Thorpe & Williams, 1941], and is hardly more specific than the possession of reducing properties.

So far as we are aware, conclusive evidence for the formation of an arylhydroxylamine derivative in the mammal has never been produced. In order to justify such a claim, it is essential that the compound, or a derivative, should be isolated in a pure condition, subjected to full elementary analysis, and compared with an authentic synthetic specimen. James's [1940] claim to have isolated *p*-hydroxylaminobenzenesulphonic acid and *p*-acetylhydroxylaminobenzenesulphonamide should be rejected on this account. Further, James states that the latter compound did not melt up to 300°, but Bratton *et al.* [1939] have synthesized the compound and shown that it has M.P. 227–229·5°. This we have confirmed [Thorpe & Williams, 1941].

There is no particular reason why biological oxidation of an aromatic amine should involve the formation of a hydroxylamine. The direct oxidation at some other position in the ring is just as feasible. In the anilines, it is known that biological oxidation takes place in the *ortho* and *para* positions to the $-NH_2$ [cf. Jaffé & Hilbert, 1888; Horn, 1936, 1, 2; 1937]. In sulphanilamide only the *ortho* or *meta* positions are available. Other work in progress in this laboratory on the metabolism of *p*-hydroxybenzenesulphonamide shows that this sulphonamide

undergoes oxidation to a small extent. It gives rise in the rabbit to small amounts of a catechol derivative, the main products being *p*-sulphonamidophenylglucuronide (isolated as Ba salt) and an ethereal sulphate. These considerations led us, *a priori*, to favour 1-amino-2-hydroxybenzene-4-sulphonamide as the oxidation product of sulphanilamide *in vivo*.

The benzoxazolone derivative (III) has been considered since benzoxazolone derivatives have been isolated from urine after feeding animals with certain aromatic amino compounds which lead to the *in vivo* formation of *o*-aminophenols, e.g. formanilide [Kleine, 1896–7], acetanilide and *o*-acetotoluidide [Jaffe & Hilbert, 1888] and salvarsan [Sieberg, 1916]. It is questionable whether these benzoxazolone derivatives were formed *in vivo* or from *o*-aminophenols during the isolation from the urines; this point is now being investigated in this laboratory. We have obtained no evidence for the formation of such a derivative from sulphanilamide. The synthesis of the compound (III) is described in the following paper [Thorpe & Williams, 1941].

In our opinion the most likely oxidation products of sulphanilamide are the aminophenolsulphonamides (IV) and (V). Such compounds would account for the increased excretion of ethereal sulphate after ingestion of sulphanilamide observed by Shelswell & Williams [1940]. We have synthesized both of these compounds. The properties of 1-amino-2-hydroxybenzene-4-sulphonamide are compatible with those of a substance in fractions obtained from urine after feeding rabbits with sulphanilamide. But our experiments on these lines are not yet complete, and we do not claim to have identified this compound. Scudi's [1940] report that he has isolated a hydroxy derivative of sulphapyridine (confirmed by elementary analysis) lends some support to our view that phenolic compounds are formed from sulphanilamide. If the formation of an *o*- or *m*-aminophenolsulphonamide could be proved, it would argue against the correctness of the hydroxylamine hypothesis, in that there is no example in the literature of the transformation of a hydroxylamine into an *o*- or *m*-aminophenol.

The indophenol test for p-*aminophenol*

This reaction has been applied to the detection of *p*-aminophenol in urines after administration of certain drugs, e.g. acetanilide, phenacetin, sulphanilamide. *p*-Aminophenol is oxidized in the presence of phenol to an indophenol dye, which is blue in alkaline and red in acid solution. We found chromic acid [Müller, 1888; Binet, 1889] a less satisfactory oxidizing agent than bleaching powder or calcium hypochlorite [Herrick & Irons, 1906].

Our interest in this test arose from claims which have been made that *p*-aminophenol could be detected and even estimated by this means in urines after administration of sulphanilamide [James, 1939; 1940]. We were of the opinion that the *in vivo* removal of the $—SO_2NH_2$ from sulphanilamide and, therefore, the formation of *p*-aminophenol was highly improbable. Since, at the time, no claim to the actual isolation of *p*-aminophenol had been made, we decided upon a critical examination of the evidence for its presence. As a preliminary, we investigated the bleaching powder variation of the indophenol reaction, and noticed that sulphanilamide itself gave a positive reaction,[1] although not so strongly as *p*-aminophenol. The reaction of the solution was of importance, the more strongly alkaline the solution the more intense being the colour. A blue colour gradually developed with *p*-aminophenol and phenol in the absence of

[1] With a freshly prepared (acid) solution of calcium hypochlorite a green colour was obtained; in the presence of NaOH or $Ca(OH)_2$ the colour was blue.

bleaching powder if the mixture was made alkaline with NaOH, Na_2CO_3 or NH_4OH, although the colours were much intensified by adding bleaching powder solution. A relatively feeble colour could be obtained by treating phenol and ammonia (without *p*-aminophenol) with calcium hypochlorite or bleaching powder solution (this is a well-known test for phenol). Such a test was obviously unsatisfactory for examination of urines containing sulphanilamide, and we have worked out a modification of the test which, while sufficiently sensitive for *p*-aminophenol, is not given by sulphanilamide. This test is performed as follows.

To 0·5–1 ml. of neutral or faintly acid test solution 3–4 drops of 5 % phenol are added, followed by a few drops of sodium hypobromite solution. With *p*-aminophenol, a blue colour appears, becoming more intense on standing and turning red on acidifying. Sulphanilamide under these conditions gives a pale pink colour and ammonium salts only a very feeble blue colour. The hypobromite solution is prepared by adding bromine to *N* Na_2CO_3 (not NaOH) until the solution is definitely yellow. It should be freshly prepared and discarded when the yellow colour has faded.

In order to detect small amounts of *p*-aminophenol in urine by this or the bleaching powder test, it is necessary to separate the *p*-aminophenol from interfering substances in the urine. This was done by two methods.

(1) *Ether extraction.* After hydrolysis with HCl (25 ml. urine boiled with 20 ml. of 3 *N* HCl for 20 min.), the urine was made faintly alkaline with Na_2CO_3 and extracted with ether in a continuous extractor for 2 hr. The indophenol test was performed on the evaporated ether extract.

(2) *Electrodialysis.* 50 ml. urine were hydrolysed by boiling for 20 min. with 1 ml. conc. HCl. The solution was neutralized with NaOH, made up to 100 ml. and placed in the middle compartment of the electrodialysis cell described by MacGregor & Thorpe [1933]. The cathode and anode compartments each held 100 ml. distilled water, and cellophane membranes were used. The cathode was of pure nickel and the anode a carbon plate. A current not exceeding 1 amp. was passed (mains voltage 220). Portions of the cathode liquid were tested at intervals. The test was regarded as negative if no indophenol reaction had been obtained after passing the current for at least 3 hr.

Using one, or more usually both, of these methods, we examined urines from eight patients receiving sulphanilamide and from twelve rabbits heavily dosed (1 g. per kg. body weight) with the drug. In no instance in which sulphanilamide was the only drug administered did we obtain a positive result. Traces of *p*-aminophenol added to urine were easily detected by either method. As a further control we dosed rabbits with acetanilide (0·1 g. per kg. body weight), which is known to be excreted partly as *p*-aminophenyl hydrogen sulphate. Good indophenol tests were obtained from these urines. We had no difficulty in detecting *p*-aminophenol in the urine of one of us who had taken a 'Veganin' tablet containing 0·25 g. phenacetin 4 hr. previously. It is known that *p*-aminophenol is excreted after taking phenacetin [Müller, 1888; Mörner, 1889]. From our experiments we can only conclude that no detectable amount of *p*-aminophenol is formed from sulphanilamide *in vivo*.

After the conclusion of our investigation, James [1940] published a paper in which he claims to have estimated and isolated *p*-aminophenol from the urine of patients receiving sulphanilamide. He states that he estimated *p*-aminophenol by the indophenol reaction. We cannot criticize this since he provides no details. His method of isolation is essentially similar to the ether extraction method which we used, and his identification, though not conclusive, is suggestive.

James's urines were presumably obtained from hospital patients. Since it is common hospital practice to permit the ward sisters to administer antipyretic drugs at their discretion, it occurred to us that James's *p*-aminophenol might be derived from phenacetin taken in such a way. We therefore worked up, according to James's directions, a 24 hr. specimen of the urine of a hospital patient who had received, in addition to sulphanilamide, some 'Veganin' tablets. We obtained an ether extract from the hydrolysed urine at *p*H 9 which gave a strong indophenol reaction.

The Pucher and Day reaction for hydroxylamines

This reaction is based on Bamberger's [1899] observation that hydroxylamine and β-arylhydroxylamines, when treated with benzoyl chloride, produce benzhydroxamic acids [see Cohen, 1931], which form violet ferric salts. Pucher & Day [1926] applied the reaction to the estimation of hydroxylamine itself, but not in urine. As a qualitative test for β-arylhydroxylamines, we believe the reaction to be reliable, the expected result being obtained after reduction by zinc dust of several nitro compounds from which the formation of hydroxylamines could be reasonably inferred. But the test is not specific, and a similar violet colour may be obtained when the test is applied to certain substances which are not hydroxylamines, even if the conditions specified by Pucher and Day for the quantitative estimation are carefully followed.

Several phenols give a violet colour with $FeCl_3$. Under the conditions of the Pucher and Day test, the phenolic hydroxyl should be benzoylated and, therefore, no violet colour would be given with $FeCl_3$. But in certain phenols another substituent group inhibits the reaction between the hydroxyl group and benzoyl chloride, so that a violet colour is produced when $FeCl_3$ is added, even the weak $FeCl_3$ used by Pucher and Day. The simplest example of such a phenol is salicylic acid, which gives a brilliant Pucher and Day reaction. Salicylamide behaves similarly. Another example is 1-amino-3-hydroxybenzene-4-sulphonic acid, which can also give a weak Pucher and Day reaction if the conditions are strictly adhered to. A more intense reaction is obtained if a stronger $FeCl_3$ solution is used (the colour from hydroxylamines is not intensified in this way), whilst with an excess of benzoyl chloride the reaction is negative. Presumably benzoylation of the hydroxyl is inhibited by the adjacent $—SO_3H$, although not to so great an extent as by —COOH or $—CONH_2$. 1-Amino-2-hydroxybenzene-4-sulphonic acid does not give the reaction, the hydroxyl being sufficiently far from the $—SO_3H$ to benzoylate without difficulty. The corresponding aminophenolsulphonamides do not give Pucher and Day's reaction. It is, therefore, essential to prove the absence of phenols which are not benzoylated before a positive Pucher and Day reaction can be taken as evidence for the presence of a hydroxylamine in a mixture of unknown composition such as urine.

We have not used the Pucher and Day test quantitatively except to make the following observation. 2 ml. of a solution of hydroxylamine hydrochloride (100 mg. per 100 ml.) were added to 4 ml. each of water and several different urines (neutral or acid to litmus). The solutions were treated according to Pucher and Day's directions for the quantitative estimation of hydroxylamine, and compared in a comparator backed in each case by the appropriate control (water or diluted urine treated with benzoyl chloride, etc.). The colour from the aqueous hydroxylamine solution was much more intense than those from the urines in most of which the colour was feeble. Further, the colours obtained from different specimens of urine were not the same. We are left with the impression that the Pucher and Day test is quite unsuitable for estimating

hydroxylamines in urines unless the hydroxylamine is previously separated from the urine.

For these reasons, we have little confidence in the report by James [1940] that he has estimated in urines, by Pucher and Day's method, the hydroxylamine formed from sulphanilamide. We are, in fact, at a loss to explain how James obtained a positive result at all. We have examined urines from eleven patients and seventeen rabbits dosed with sulphanilamide and have only once obtained a positive reaction.[1] This urine was from a patient who had also taken some acetylsalicylic acid in the form of 'Veganin' tablets. The presence of salicylic acid in this urine was detected by the formation of methyl salicylate. The urine gave a violet colour with $FeCl_3$ alone. James does not record that he tested his urines for salicylates. If the Pucher and Day test is claimed as evidence for the presence of hydroxylamine in human urine, the claim should at least be accompanied by the assurance that the urine gave no colour with $FeCl_3$ alone.

Marshall's diazo reaction applied to sulphanilamide and β-arylhydroxylamines

Sulphanilamide is usually estimated in blood and urine by diazotization with $NaNO_2$ and HCl. After addition of ammonium sulphamate to remove excess HNO_2, the solution is coupled with an alcoholic solution of a naphthylamine derivative, e.g. dimethyl-α-naphthylamine [Marshall *et al.* 1937], and the resulting red colour matched in a colorimeter. Rosenthal & Bauer [1939] called attention to Bamberger's [1898] observation that β-arylhydroxylamines form diazonium salts with nitrous acid, and showed that while the amino group was readily acetylated with acetic anhydride and thus rendered undiazotizable, β-arylhydroxylamines could be diazotized even after treatment with acetic anhydride.[2] They therefore estimated the red diazo colour before and after treatment with acetic anhydride. They claimed that the difference gave the true sulphanilamide value, and that the value obtained after acetylation was due to arylhydroxylamine.

We have tested Rosenthal and Bauer's hydroxylamine reaction by reducing a number of aromatic nitro compounds with zinc dust. The solutions readily diazotized and coupled even after treatment with acetic anhydride and the presence of a hydroxylamine in the solutions was confirmed by the Pucher and Day test. But we also obtained results which led us to doubt the reliability of these tests, and, therefore, their quantitative value in solutions of uncertain composition such as urine.

It is not justifiable to assume that sulphanilamide and *p*-hydroxylaminobenzenesulphonamide are the only compounds present in the urine of a subject who has received sulphanilamide which diazotize and couple. There is the possibility of the presence of a diazotizable aminophenol derivative such as 1-amino-3-hydroxy- or 1-amino-2-hydroxy-benzenesulphonamide. We have, in fact, some evidence that such a compound is present in sulphanilamide urines. If *p*-aminophenol were formed from sulphanilamide—which we do not believe—

[1] To eliminate the possibility of the hydroxylamine being destroyed on keeping in urine, we tested some urines from rabbits and one human urine within 15 min. of the sample being voided. No positive results were obtained.

[2] The chemistry of this reaction is far from clear. If an arylhydroxylamine is acetylated it should not diazotize and couple. Bratton *et al.* [1939] have stated, and we have confirmed, that *p*-*N*-acetylhydroxylaminobenzenesulphonamide does not diazotize and couple. (See also Thorpe & Williams [1941].)

it would be partly excreted as *p*-aminophenylsulphuric acid, which can be diazotized and will then couple with dimethyl-α-naphthylamine [James, 1940]; free *p*-aminophenol only does so in concentrated solutions. 1-Amino-2-hydroxybenzene-4-sulphonamide [Thorpe & Williams, 1941], 1-amino-2-hydroxybenzene-4-sulphonic acid [Friedländer, 1911], *o*-aminophenol and 1-hydroxy-2-aminobenzene-4-sulphonic acid (B.D.H.) give, with nitrous acid, a yellow colour which does not become red on adding dimethyl-α-naphthylamine. In all these compounds there is an OH group *ortho* to the NH_2 and diazo-oxide formation is possible [see Hickinbottom, 1936]. If, however, this OH is methylated the red colour is obtained. Methylated solutions of the above phenols were prepared by shaking their aqueous solutions with methyl sulphate and alkali at room temperature: in the case of *o*-aminophenol, *o*-anisidine was used in addition to the methylated aqueous solution of the phenol itself. It appears, therefore, that if any 1-amino-2-hydroxybenzene-4-sulphonamide were formed *in vivo* and excreted conjugated with glucuronic acid and/or sulphuric acid at the OH group, then such conjugates would give the Marshall diazo reaction in addition to sulphanilamide itself.

In certain compounds, a diazo reaction after acetic anhydride treatment can be due to a free amino group, since other groups present may prevent the acetylation of the $—NH_2$. The first example of this which we encountered was 1-nitro-3-aminobenzene-4-sulphonic acid, which cannot be acetylated under the Rosenthal and Bauer conditions, even if a large excess of acetic anhydride be used, so that the 'acetylated' solution of this compound gives a diazo reaction. A similar result might be expected with an *o*-aminophenol conjugated through its —OH with sulphuric or glucuronic acids (see next paragraph), e.g. 1-amino-2-hydroxybenzene-4-sulphonamide, which is a possible detoxication product of sulphanilamide. In a note published some time after Rosenthal and Bauer's paper, Rosenthal [1939, 2] withdrew their claim for specificity and quoted sulphanilamide glucoside (structure not specified) as the compound giving the reaction.

We have examined twenty-four urines (human and rabbit) from subjects treated with sulphanilamide and obtained a positive diazo reaction after acetylation in thirteen cases, eight being very feeble. In none of these urines has the suggestion of an arylhydroxylamine been supported by a positive Pucher and Day test. It can be argued that the diazo test is more sensitive than that of Pucher and Day, and that, therefore, these observations are not incompatible with the presence of an arylhydroxylamine. Against this is the following observation. The only strong reactions were obtained in rabbit urines (presumably owing to the relatively higher dosage). If such urines were boiled with dilute HCl for a few minutes, a diazo reaction after acetylation was no longer given (a solution of *p*-hydroxylaminobenzenesulphonamide still gave the acetylated diazo reaction after boiling with HCl under similar conditions). Such a result could be due to an *o*-aminophenol derivative, e.g. 1-amino-2-hydroxybenzene-4-sulphonamide excreted in conjugation with sulphuric acid. The close proximity of the strongly acidic $—SO_4H$ group to the amino group might prevent acetylation of the latter, and the compound could, therefore, give a Rosenthal and Bauer diazo reaction. After hydrolysis there would be no diazo reaction because 1-amino-2-hydroxybenzene-4-sulphonamide does not couple. In support of this is the fact that the urine before hydrolysis gave no colour with $FeCl_3$, whereas after hydrolysis it gave a red-violet colour.

From the foregoing it is clear that neither the Pucher and Day nor the Rosenthal and Bauer tests can be relied upon for the detection of hydroxylamines

in urine. In the absence of a specific reaction, we suggest that the following tests should be applied to the urine.

Test	Expected result if arylhydroxylamine is present
1. Pucher and Day test	Violet colour
2. Marshall's diazo reaction	Red colour
3. Diazo reaction after acetylation (Rosenthal and Bauer)	Red colour
4. Repeat test 2 on the urine after short boiling with HCl	Red colour
5. Add $FeCl_3$ solution	No red or violet colour
6. Repeat test 5 on the urine, after boiling with HCl and neutralizing	No red or violet colour

If *all* these tests give the expected results, there is a probability, but not a certainty, that a β-arylhydroxylamine is present. Tests 2 and 3 would not be given by an arylacetylhydroxylamine. With β-phenylhydroxylamine the result of test 6 is abnormal owing to the formation of *p*-aminophenol. Table 1 gives some typical results with these tests.

Table 1

	Pucher and Day 1	Marshall's diazo 2	Rosenthal and Bauer diazo 3	Marshall's diazo after HCl 4	$FeCl_3$ 5	$FeCl_3$ after HCl 6
β-Phenylhydroxylamine	+	+ *	+	+	−	+
p-Hydroxylaminobenzenesulphonamide	+	+	+	+	−	−
p-Acetylhydroxylaminobenzenesulphonamide	+	−	−	+	+	−
Salicylic acid	+	−	−	−	+	+
Salicylamide	+	−	−	−	+	+
1-Amino-2-hydroxybenzene-4-sulphonic acid	−	−†	−	−	+	+
1-Amino-2-hydroxybenzene-4-sulphonamide	−	−†	−	−	+	+
1-Amino-3-hydroxybenzene-4-sulphonic acid	+	+	−	+	+	+
1-Amino-3-hydroxybenzene-4-sulphonamide	−	+	−	+	+	+
1-Nitro-3-aminobenzene-4-sulphonic acid	−	+	+	+	−	−
1-Nitro-3-aminobenzene-4-sulphonamide	−	+	+	+	−	−
2-Oxybenzoxazole-6-sulphonic acid	−	−	−	−	−	−
2-Oxybenzoxazole-6-sulphonamide	−	−	−	−	−	−
p-Aminophenol	−	+‡	−	+‡	+	+
Sulphanilamide	−	+	−	+	−	−

\+ Indicates expected colour obtained. − No colour.

* Excess of nitrite may be required to get good reaction.
† Positive after methylation. ‡ Colour feeble and bluish.

Summary

The evidence for the biological formation of arylhydroxylamines is reviewed. It is concluded that there is as yet no reliable evidence for the biological formation of such compounds in the mammal, and, in particular, for the presence of *p*-hydroxylaminobenzenesulphonamide in the urine of humans and rabbits treated with sulphanilamide.

Whilst *p*-aminophenol can be detected in urine after treatment with certain drugs (e.g. acetanilide, phenacetin), there is no reliable evidence that it occurs in urine after treatment with sulphanilamide.

The colour reactions (Rosenthal and Bauer's diazo test, Pucher and Day's test, and the indophenol test) which have been used for identification of these substances have been examined and shown unreliable for this purpose.

We wish to thank Dr A. R. McWhinney, Dr G. E. Murphy, Miss J. Pitt, Mr Garfield Thomas and Dr Margaret Thorpe for obtaining for us specimens of urine from sulphanilamide-treated patients.

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9. STUDIES IN DETOXICATION

9. THE SYNTHESIS OF SOME POSSIBLE BIOLOGICAL OXIDATION PRODUCTS OF SULPHANILAMIDE

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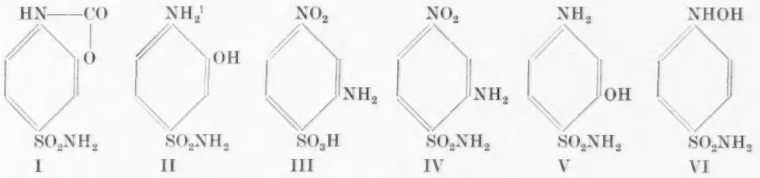
In the previous paper [Thorpe & Williams, 1941] we expressed the opinion that 1-amino-2-hydroxy-(II) and 1-amino-3-hydroxy-benzene-4-sulphonamide (V) and 2-oxybenzoxazole-6-sulphonamide (I) might be biological oxidation products of sulphanilamide. Here we describe the synthesis of these compounds. No difficulty was experienced in the preparation of the *o*-aminophenol and benzoxazole derivatives which were obtained at different stages in the same synthesis, but the synthesis of the *m*-aminophenol derivative presented unexpected difficulties:



We first attempted a synthesis of 1-amino-3-hydroxybenzene-4-sulphonamide (V), starting with the easily prepared *m*-nitraniline-sulphonic acid (III). It was hoped to obtain large amounts of 1-nitro-3-aminobenzene-4-sulphonamide (IV), and thence, by diazotization and reduction, the required *m*-aminophenolsulphonamide. We found, however, after many trials using different chlorinating agents, that the nitranilinesulphonic acid formed little or none of the corresponding sulphonyl chloride. Small amounts of (IV) were eventually obtained in one experiment using chlorosulphonic acid, but this result could not be repeated. This proposed method of synthesis was therefore abandoned. One interesting point which emerged at this stage was that ammonium 1-nitro-3-aminobenzene-4-sulphonate was isolated in a dimorphous state, a pale orange and a red form. The orange form could be easily converted into the red form by gentle warming below 100°. The red form, once formed, was apparently stable.

Success was eventually attained starting with *m*-aminophenolsulphonic acid, which is easily prepared by heating *m*-aminophenol with conc. H_2SO_4. The synthesis worked smoothly to the sulphonyl chloride stage, whilst the final stages were completed with some difficulty.

Although many workers (see previous paper for literature) state that they have used *p*-hydroxylaminobenzenesulphonamide (VI) in biological work, no mention is made in their papers of its method of preparation or chemical properties. This substance has been prepared by Bratton *et al.* [1939] and we have repeated their process and agree with their description of its chemical properties.

We have also prepared its *N*-acetyl derivative[1] which has M.P. 227° and gives no diazo reaction in agreement with Bratton *et al.* [1939]. We mention this since James [1940] claims to have isolated this acetyl compound from human urine after sulphanilamide medication, and states that the substance he isolated did not melt below 300°. We failed to prepare *p*-hydroxylaminobenzenesulphonamide using Bamberger's [1894] method with zinc dust at low temperatures, and Haworth & Lapworth's [1921] sodium hydrosulphide method for the preparation of arylhydroxylamines.

The phenolic sulphonamides (II) and (V) have not yet been tested for therapeutic activity. Of phenolic sulphonamides which have been tested for therapeutic activity, 1-hydroxy-2-aminobenzene-4-sulphonamide [Tréfouel *et al.* 1937; McLeod, 1938] and *p*-hydroxybenzenesulphonamide [Buttle *et al.* 1936; McLeod, 1938] were inactive, and 1-hydroxy-2-nitrobenzene-4-sulphonamide [McLeod, 1938] was active. Compounds related to our 1-nitro-3-aminobenzene-4-sulphonamide have also been tested and found inactive, namely 1-nitro-4-aminobenzene-3-sulphonic acid [Buttle *et al.* 1936] and 1-nitro-6-aminobenzene-3-sulphonamide [Tréfouel *et al.* 1937].

Experimental

2-Oxybenzoxazole-6-sulphonyl chloride. Benzoxazolone (2-oxybenzoxazole) was prepared from salicylamide and NaOCl according to Graebe & Rostovzeff [1902]. The only modification introduced was that the benzoxazolone was precipitated from the alkaline reaction mixture by neutralizing carefully, with cooling, with conc. HCl instead of by passing CO_2 into the solution. The neutralization with CO_2 may take several hours (on a 20 g. scale) whilst with HCl it can be done in under half an hour. Sodium benzoxazolonesulphonate was prepared according to the patent directions [Friedländer, 1911]. The dry salt (10 g.) was stirred with 12 g. PCl_5. The mass warmed up and became pasty, and after standing half an hour with occasional stirring, it was treated with ice water in the usual manner. The precipitated *chloride* was filtered, washed with water and dried (yield 6 g.). It crystallized from toluene in colourless rhombic plates, M.P. 186–187°.[2] (Found: C, 36·2; H, 1·8; N, 6·1 %. $C_7H_4O_4NClS$ requires C, 36·0; H, 1·7; N, 6·0 %).

2-Oxybenzoxazole-6-sulphonamide (I) was prepared by adding the powdered chloride to strong ammonia. The deposit formed overnight was filtered and dissolved in warm dilute NaOH. On cooling, the *sodium salt* of the sulphonamide separated as needles, which were recrystallized from water. (Found: N, 10·2; Na, 8·4; H_2O, 13·4 %. $C_7H_5O_4N_2SNa$, $2H_2O$ requires N, 10·3; Na, 8·45; H_2O, 13·2 %.) The *sulphonamide* itself immediately separates as needles if an aqueous solution of the sodium salt is acidified. On recrystallization from water, the sulphonamide formed needles, M.P. 263°. (Found: C, 39·4; H, 2·8; N, 13·3 %. $C_7H_6O_4N_2S$ requires C, 39·25; H, 2·8; N, 13·1 %.)

1-Amino-2-hydroxybenzene-4-sulphonamide (*II*). 2·8 g. of the foregoing sodium salt were boiled for 20 min. with 25 ml. of 30 % NaOH. Crystals of

[1] We experienced some difficulty in this acetylation. Using acetic anhydride as recommended by Bratton *et al.* we persistently obtained a product melting about 160° from which Bratton's acetyl derivative was only obtained in small yield by fractional crystallization. We did not examine the other product in detail except to note that it gave a diazo reaction, and prove that it was not unchanged *p*-hydroxylaminobenzenesulphonamide. It may be the substance responsible for Rosenthal and Bauer's diazo reaction (see Thorpe & Williams [1941]).

[2] All melting-points are uncorrected.

Na_2CO_3 separated in the solution, and were liable to cause violent bumping. The solution was cooled, carefully neutralized with conc. HCl and then made alkaline with solid Na_2CO_3. The solution was now extracted for 8 hr. with ether in a continuous extractor and on evaporation of the ether the *sulphonamide* was obtained as a crystalline residue (2 g.). The compound was purified by dissolving in hot absolute alcohol and adding sufficient carbon tetrachloride to produce a faint cloudiness; on cooling the sulphonamide separated as clusters of long needles, M.P. 164°. It was soluble in water and alcohol, sparingly soluble in ether and insoluble in CCl_4. It reduced ammoniacal silver nitrate on warming and gave a purple colour with $FeCl_3$ turning a deep red in about 30 sec. It slowly caused the formation of methaemoglobin in a 1 in 50 solution of blood at 37°. (Found: C, 38·6; H, 4·3; N, 15·6 %. $C_6H_8O_3N_2S$ required C, 38·3; H, 4·3; N, 14·9 %.) The *hydrochloride* was prepared by adding the requisite amount of conc. HCl to an alcoholic solution of the sulphonamide. On stirring the solution the hydrochloride separated; more alcohol was now added and the solution boiled until the hydrochloride dissolved. Carbon tetrachloride was then poured into the solution until the hydrochloride just began to crystallize and the crystallization was completed at 0°. It formed fine matted needles, which were very soluble in water and which decomposed without melting above 300°. It was purified by crystallization from absolute alcohol and CCl_4. (Found: C, 32·3; H, 3·9; N, 12·6 %. $C_6H_8O_3N_2S.HCl$ requires C, 32·1; H, 4·0; N, 12·5 %.)

Treatment of an aqueous solution of the sulphonamide with benzoyl chloride and NaOH gave *1-benzamino-2-benzoxybenzene-4-sulphonamide*, which formed needles from aqueous alcohol, M.P. 191°. (Found: C, 60·0; H, 3·9; N, 7·4 %. $C_{20}H_{16}O_5N_2S$ requires C, 60·6; H, 4·1; N, 7·1 %.)

1-Amino-3-hydroxybenzene-4-sulphonic acid was prepared by sulphonation of *m*-aminophenol [cf. Friedländer, 1896]. 15 g. of *m*-aminophenol were heated on the water bath with 25 ml. conc. H_2SO_4 and a few drops of fuming H_2SO_4 for an hour. The solid product was broken up in about 150 ml. of water and 10 % NaOH added with stirring until a clear solution was obtained. The solution was now filtered, acidified with HCl and set aside to crystallize at 0°. The acid (tablets) was filtered, washed with water and dried over $CaCl_2$; yield 24 g.

Pyridine 1-acetamino-3-acetoxybenzene-4-sulphonate. The above acid (10 g.) was shaken with 25 ml. pyridine and then 25 ml. acetic anhydride added. Addition of the anhydride caused the evolution of heat; the solution was therefore cooled under the tap. The acid dissolved in about an hour, the solution being occasionally shaken to facilitate solution. The mixture solidified on keeping at room temperature overnight. The solid was filtered under suction, pressed and washed with small amounts of methylated spirit. It was recrystallized from spirit, ether being added to facilitate the crystallization. On evaporating the mother liquor from the acetylation mixture *in vacuo*, a small amount of the salt was recovered; yield 15 g. M.P. 170–172°. The substance gave no colour with $FeCl_3$ and no diazo reaction. (Found: N, 7·9 %. $C_{15}H_{16}O_6N_2S$ requires N, 7·95 %.)

1-Acetamino-3-acetoxybenzene-4-sulphonyl chloride. The foregoing pyridine salt (5 g.) was stirred with 5 g. PCl_5. The mixture soon became pasty and warm. After 15–20 min., the mixture was heated for a few minutes on the water bath. The product was then thrown into ice-water and, on stirring, the *chloride* separated as a white solid (yield almost quantitative). It crystallized from toluene as long rods, M.P. 169°. (Found: C, 41·4; H, 3·4; N, 4·85 %. $C_{10}H_{10}O_5NClS$ requires C, 41·2; H, 3·45; N, 4·8 %.)

1-Acetamino-3-hydroxybenzene-4-sulphonamide. The above chloride was added to 5 times its weight of 40 % ammonia (40 ml. NH_4OH sp.gr. 0·880 made

up to 100 ml. with water). The chloride gradually dissolved, the solution becoming warm. After an hour the opalescent solution was filtered and evaporated to a thin paste on the water bath. After cooling, the paste was stirred with about 5 vol. water and the sparingly soluble crystalline material collected and washed with water. The *1-acetamino-3-hydroxybenzene-4-sulphonamide* was recrystallized from 50 % aqueous alcohol as colourless needles, M.P. 280° decomp. (The melting-point was observed after raising the temperature fairly rapidly up to about 270°; if heated very slowly decomposition sets in about 250°, observed melting-point may be as low as 265° and it is difficult to get consistent results.) Yield of crude product 30 % weight of chloride. The pure substance gave no diazo reaction. A violet colour with $FeCl_3$ was obtained with a solution of the amide in 50 % aqueous alcohol. (Found: C, 41·8 %; H, 4·5; N, 12·2 %. $C_8H_{10}O_4N_2S$ requires C, 41·75; H, 4·4; N, 12·2 %.)

The mother liquor from the separation of this amide gave on further evaporation crystalline material (yield up to 10 % weight of chloride) rather more soluble than the main product. It crystallized from 30 % aqueous alcohol in apparently homogeneous needles, M.P. 235°. It gave no diazo reaction, but readily gave a violet colour with $FeCl_3$ with an aqueous solution. On hydrolysis with 2*N* NaOH, it yielded 1-amino-3-hydroxybenzene-4-sulphonamide. C, H and N values (C, 42·6; H, 4·5; N, 11·1 %) gave no clue to the nature of this substance (mixture?) and we did not investigate it further.

The filtrate from the separation of the unidentified substance appeared to contain small amounts of this unidentified substance or the above amide (or both) and also some acetylated 1-amino-3-hydroxybenzene-4-sulphonic acid, since after hydrolysis with 2*N* NaOH some 1-amino-3-hydroxybenzene-4-sulphonamide could be obtained by extraction with ether, and 1-amino-3-hydroxybenzene-4-sulphonic acid (yield up to 10 % weight chloride) separated in tablets on acidifying with HCl.

1-Amino-3-hydroxybenzene-4-sulphonamide (*V*). 1-Acetamino-3-hydroxybenzene-4-sulphonamide (1 g.) was dissolved in 10 ml. 2*N* NaOH and heated in a boiling water bath for 3½ hr. Water lost by evaporation was replaced at intervals. (Hydrolysis with 2*N* HCl was equally effective.) The mixture was then made neutral or just acid by addition of conc. HCl and finally rendered just alkaline by addition of solid Na_2CO_3. The solution was extracted with ether in a continuous extractor for at least 8 hr. *1-Amino-3-hydroxybenzene-4-sulphonamide* separated from the extract in clusters of long colourless plates (yield 0·5 g.). A further crop (0·1 g.) was obtained by evaporation of the ether, but the product obtained in this way was liable to turn brown on keeping unless it was immediately purified. The amide was recrystallized by taking up in a small amount of absolute alcohol followed by addition of CCl_4 until cloudy. The amide gradually crystallized in long plates which had M.P. 152° and gave a strong diazo reaction and a weak red-violet colour with $FeCl_3$. It was readily soluble in water and alcohol, sparingly soluble in ether, insoluble in CCl_4. (Found: C, 38·7; H, 4·4; N, 14·6 %. $C_6H_8O_3N_2S$ requires C, 38·3; H, 4·3; N, 14·9 %.) On adding conc. HCl to a concentrated aqueous solution, the *hydrochloride* separated in elongated rhombic plates which could be recrystallized from a small volume of 80 % aqueous alcohol. By shaking an aqueous solution of the amide with acetic anhydride, the monoacetyl derivative M.P. 280° already described separated from the solution. The amide did not cause formation of methaemoglobin when incubated with a 1 in 50 blood solution.

1-Nitro-3-aminobenzene-4-sulphonamide (*IV*). *m*-Nitraniline was sulphonated according to Friedländer [1923] and 1-nitro-3-aminobenzene-4-sulphonic acid isolated as the sparingly soluble sodium salt. Treatment of the dry Na salt or of

the free acid itself with PCl_5, thionyl chloride or chlorosulphonic acid failed to give any indication of the formation of the sulphonyl chloride except in one case. An account of this isolated case is given, but we must emphasize that this experiment could not be repeated. 20 g. of the dry *m*-nitranilinesulphonic acid were heated on the water bath for 3·5 hr. with 20 ml. of chlorosulphonic acid. The resulting thick liquid was poured into ice-water with stirring. A yellow precipitate separated which was filtered and washed twice with water. After pressing as dry as possible, it was stirred into 200 ml. ammonia (sp. gr. 0·880), in which it immediately dissolved. After 24 hr. at room temperature a deposit (6 g.) of clear light brown prisms of large size separated, having M.P. 213–214°. On evaporating the mother liquor *ammonium 1-nitro-3-aminobenzene-4-sulphonate* was obtained. This salt crystallized at room temperature as large orange prisms, but if the temperature of crystallization was above room temperature, then a mixture of bright red and orange crystals was obtained. The orange form could be easily converted into the red form on heating in a test tube at about 80°. The red form once formed appeared to be stable. (Found, orange form: C, 30·8; H, 3·8; N, 17·6 %: red form, C, 30·5; H, 3·8; N, 17·8 %. $C_6H_9O_5N_3S$ requires C, 30·6; H, 3·9; N, 17·9 %.) The light brown crystals of M.P. 213–214° appeared to be mainly *1-nitro-3-aminobenzene-4-sulphonamide*, contaminated with some of the above ammonium salt. The crystals were recrystallized from water and formed yellow prismatic needles, M.P. 215°. (Found: C, 33·3; H, 3·2 %. $C_6H_7O_4N_3S$ requires C, 33·2; H, 3·25 %.)

Summary

The synthesis of 1-amino-2-hydroxybenzene-4-sulphonamide, 2-oxybenzoxazole-6-sulphonamide, 1-amino-3-hydroxybenzene-4-sulphonamide and several related compounds is described. Their preparation was necessitated because they may be biological oxidation products of sulphanilamide.

1-Amino-2-hydroxybenzene-4-sulphonamide is a methaemoglobin-former *in vitro*, but 1-amino-3-hydroxybenzene-4-sulphonamide is not.

We wish to express our gratitude to Prof. W. N. Haworth and Dr M. Stacey, who arranged for the C, H and some of the N estimations to be done by their micro-analyst.

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10. PRODUCTION OF AMINES BY BACTERIA

4. THE DECARBOXYLATION OF AMINO-ACIDS BY ORGANISMS OF THE GROUPS *CLOSTRIDIUM* AND *PROTEUS*

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The previous papers of this series have dealt with the production of amines by strains of *Bact. coli* [Gale, 1940, 1] and *Strep. faecalis* [Gale, 1940, 2, 3] and with the properties of the amino-acid decarboxylases involved. The present communication continues the study for members of the strictly anaerobic group of *Clostridia*. Results obtained with members of the *Proteus* group are also included, but it will appear that these organisms are not important amine-producers.

Kendall & Schmidt [1926] found that cultures of *Cl. welchii*, when grown in a medium containing glucose, produce a substance in the medium which has the physiological reactions of histamine. Later Kendall & Gebauer [1930] isolated a substance from a bulk culture which had the chemical properties of histamine. Eggerth [1939], in his investigations on the production of histamine by bacteria in culture, obtained positive results with four strains of *Cl. welchii* and most accounts of the toxins of this organism now mention the formation of a 'non-specific histamine-like toxin'. Apart from these instances there appears to be no mention of amine-production by *Clostridia* in the literature. The amino-acid metabolism of certain of the strict anaerobes has been studied: *Cl. sporogenes* by Stickland [1934; 1935], Woods [1936], Kocholaty & Hoogerheide [1938]; *Cl. tetanomorphum* by Woods & Clifton [1937; 1938], and *Cl. botulinum* by Clifton [1940].

Methods

The general technique of preparation of washed suspensions, determination of their dry weight and the manometric investigation of their power to decarboxylate amino-acids has been described fully in the previous papers [Gale, 1940, 1, 2]. In the present survey, the organisms were grown overnight in 800 ml. 2% glucose broth (tryptic digest of casein), the total organisms obtained made into washed suspension and tested against the 16 amino-acids previously described; for the first experiment in each case, washed suspensions containing 20–30 mg. bacteria/ml. were used so that small activities could be detected, and the suspensions later diluted to extents indicated by the preliminary test. The optimum *p*H was determined in every case and the experiment carried out, unless otherwise stated, at a thermostat temperature of 30°. Decarboxylase activity is measured in terms of Q_{CO_2} as before and values <1 are regarded as not significant.

The *Clostridia* were kept in most cases in tubes of broth and heart muscle. In the case of *Cl. welchii* a 'cellar' of alkaline egg medium was also maintained, the organism being subcultured into meat-broth before use. It was found,

[1] Senior Student of the Royal Commission for the Exhibition of 1851.

however, that the organism lost activity after 5–6 serial cultivations in broth and so cultures were taken afresh from the egg medium once a week. For experimental purposes the medium used consisted of tryptic digest of casein with a little heart muscle and 2% additional glucose. This was inoculated with the contents of a cultured tube and incubated at 37° overnight in a Fildes and MacIntosh anaerobic jar. In all cases the organisms were examined microscopically before use to determine the extent to which sporing had occurred; in most cases cultures of 12–15 hr. consisted almost entirely of vegetative cells, exceptions being *Cl. septique* and *Cl. tetanomorphum* where considerable numbers of spores were sometimes encountered. Most of the *Clostridia* grow very thickly in the medium described, growth ceasing after 8–12 hr. when the culture has reached 0·75–0·95 mg. dry weight of bacteria/ml. medium in many cases. *Cl. septique* again forms an exception in that the onset of sporing seems to limit the growth to approx. 0·3 mg./ml. On harvesting, the culture was poured through glass wool to remove fragments of meat and then centrifuged and washed as usual. A few strains of *Cl. welchii* proved difficult to centrifuge cleanly owing to 'roping' of the culture, but this difficulty was only marked with the strains S.R. 9 and Adams, in which cases it was necessary to rely on serial concentration of the suspension rather than on clean separation of the organisms. All washed suspensions were used on the day on which they were prepared, but it was not found necessary to take any special measures to keep them active with regard to the decarboxylases over a period of 4–7 hr.

Organisms and strains used

Cl. welchii S.R. 9. Classical strain used for toxin production. Isolated from war wound. Toxic type A.

Cl. welchii T.W. 14. Isolated from case of puerperal fever. Type A.

Cl. welchii Chelsea. Probably atoxic strain isolated from the urine of a pregnant woman.

Cl. welchii Fisher. Isolated from war wound. Type A.

Cl. welchii Adams. Very toxic strain from war wound. Type A.

Cl. welchii B.W. 21. Isolated from war wound by Prof. MacIntosh. Type A.

Cl. welchii S. 107. Isolated from sheep faeces. Type A.

Cl. paludis Wye 4. *Welchii*-type organism causing sheep disease. Type B.

Lamb dysentery U. 15. *Welchii*-type organism. Type C.

Cl. septique Pasteur III. Original strain isolated by Pasteur.

All the above organisms and also *Cl. septique* Stokes and Jerral were obtained from Dr M. Robertson to whom my thanks are due for advice on the maintenance of these cultures. I am indebted to Dr Carruthers of the Emergency Public Health Laboratory Service for strains of *Cl. septique*, the non-toxigenic strain of *Cl. tetanum* Fildes and for the various *Proteus* organisms mentioned. All other organisms mentioned below were obtained from the National Collection of Type Cultures and are referred to by their collection number.

Decarboxylation of amino-acids by organisms of the groups Clostridium *and* Proteus

The general conditions under which certain amines are produced by bacteria have been elucidated in the previous papers of this series. In the present work it has been the custom first to undertake a survey of the decarboxylating activities of several strains of each organism when grown in glucose broth and then to choose the most active strain for detailed investigation. As a result of this survey with the two groups of organisms it has been found that only four amino-

acids are attacked under the usual experimental conditions and Table 1 shows the activities of the various positive organisms. In each case the Q_{CO_2} value is given for the activity at the optimal *p*H (see below) and 30° and represents the initial steady velocity of simple decarboxylation of the amino-acid in question. The decarboxylation is quantitative in every case since the theoretical volume of CO_2 is obtained within experimental error on completion; the decarboxylation is not accompanied by any other reaction so that the product is the amine corresponding to the amino-acid substrate and this is confirmed by isolation and identification of the product as shown later. For this preliminary work the organisms were grown overnight in the 2 % glucose broth medium described and the *p*H of the medium measured potentiometrically before harvesting as a measure of the saccharolytic activity of the organisms concerned.

Table 1. *Decarboxylation of amino-acids by various* Clostridium *and* Proteus *organisms grown in 2 % glucose broth at 37°. Activities expressed as* Q_{CO_2} *obtained with washed suspensions at 30° and the optimum* p*H in each case*

Organism	Strain	Final *p*H in medium	Q_{CO_2} Histidine	Ornithine	Tyrosine	Glutamic acid
Cl. welchii	S.R. 9	4·6	34	—	—	70
Cl. welchii	T.W. 14	4·5	3	—	—	237
Cl. welchii	Chelsea	4·5	30	—	—	368
Cl. welchii	Fisher	4·6	29	—	—	100
Cl. welchii	Adams	4·6	27	—	—	116
Cl. welchii	B.W. 21	4·1	60	—	—	132
Cl. welchii	S. 107	4·2	23	—	—	50
Cl. paludis	Wye 4	4·6	26	—	—	36
Lamb dysentery	U. 15	4·6	6	—	—	40
Cl. septique	Carr.	5·3	—	180	—	—
Cl. septique	Pasteur	5·3	—	270	—	—
Cl. septique	Stokes	6·0	—	176	—	—
Cl. septique	Jerral	5·75	—	70	—	—
Cl. aerofoetidum	505	5·3	—	—	50	325
Cl. fallax	Morcom	5·1	14	—	—	—
Cl. fallax	2907	5·05	—	—	—	—
Cl. bifermentans	2914	5·2	8	—	—	80
Cl. bifermentans	536	5·4	—	—	—	30
Pr. vulgaris	Fildes	5·25	—	—	—	324
Pr. vulgaris	Crook	5·63	—	9	—	112
Pr. vulgaris	Carr.	4·85	—	2	—	46
Pr. morganii	Carr.	5·25	—	12	—	134
Pr. morganii	Carr.	5·8	—	16	—	—

Proteus organisms HX 2, HXL 11, HX 19, etc., also attacked glutamic acid, while Kingsbury strains apparently possessed no decarboxylating enzymes. The following members of the *Clostridium* group showed no decarboxylating activities under the usual experimental conditions:

Cl. sporogenes Bellette 533 and 2911.
Cl. histolyticum Weinberg and 6282 (American).
Cl. chauvoei 287 and 2721.
Cl. oedematiens (*novyi*) 2908 and Cossard 277.
Cl. tetanum Fildes.
Cl. butyricum Weizmann 619.
Cl. tertium 336.
Cl. multifermentans-tenalbus 2032.
Cl. tetanomorphum 500 and 2909.
Cl. sphenoides 507.

The organisms chosen for detailed investigation were *Cl. welchii* B.W. 21, *Cl. septique* Pasteur III, *Cl. aerofoetidum* 505, *Cl. fallax* Morcom, *Cl. bifermentans* 2914 and *Proteus morganii*; the results set out below were obtained chiefly with these selected strains.

Properties of the amino-acid decarboxylases

l(−)-*Histidine decarboxylase.* Kendall & Schmidt [1926] reported that 66 strains out of 72 of *Cl. welchii* tested produced a histamine-like substance when grown in the presence of glucose. In Table 1 it can be seen that all the strains used in washed suspension by the author produce histamine under the appropriate conditions. One strain, not included in Table 1, attacked glutamic acid rapidly but failed to decarboxylate histidine, but since this strain has also proved abnormally erratic in its power to produce specific toxin, it has been omitted. Kendall & Schmidt state that certain of their strains lost the power to produce histamine on continued subcultivation and it has been mentioned above that the author found it necessary to return to the alkaline egg medium once a week in order to maintain activity. Histidine is also decarboxylated, but at a slower rate, by some strains of *Cl. fallax* and *Cl. bifermentans*. Gale [1940, 1] showed that strains of *Bact. coli* in washed suspension will decarboxylate histidine quantitatively to histamine at an optimal *p*H of 4·0. When testing the power of *Cl. welchii* to carry out this reaction at various *p*H values, it became obvious that 4·0 is not the optimum *p*H in this case. Fig. 1 shows the variation of histidine decarboxylase activity with reaction *p*H for strains of *Cl. welchii*, *Cl. fallax* and *Cl. bifermentans*. The optimal activity is obtained between *p*H 2·5 and 3·0 in all cases: *Cl. welchii* B.W. 21 appears to have a well-defined optimum at 2·5 while that of the S. 107 strain lies nearer 3·0. The Q_{CO_2} figures for *p*H values <2 can only be regarded as approximate as the HCl-phthalate mixtures used are not very effective buffers over this part of the range and calculations were accordingly made over a short period of activity, the *p*H being measured immediately before and after this period. The *p*H-activity curve is markedly different from that obtained for the decarboxylation of histidine by *Bact. coli* [Gale, 1940, 1] which has an optimum at *p*H 4·0 in all cases tested. The curve obtained with the coliform organisms falls steeply on the acid side of *p*H 4·0, the enzyme being almost inactive at *p*H 3·5 and it has been suggested that this is due to denaturation of the enzyme protein while in actual practice it is often found that the organisms coagulate below *p*H 4·0. This is not the case with *Cl. welchii* where coagulation of the organisms does not take place even at *p*H 1 and the *p*H-activity curve for the histidine decarboxylase is approximately symmetrical. Thus the difference in the apparent properties of the two enzymes may be due to the greater resistance to denaturation of the proteins of *Cl. welchii* over those of the coliform organisms, but the question is not likely to be solved until both enzymes are obtained in a cell-free state.

l(+)-*Ornithine decarboxylase.* Ornithine is decarboxylated rapidly by all four of the *Cl. septique* strains tested and much more slowly by *Proteus morganii* and some strains of *Pr. vulgaris*. Fig. 2 shows the variation of ornithine decarboxylase activity of some of these three organisms with reaction *p*H. The *Cl. septique* strains show a well-marked optimum at *p*H 5·5, the most alkaline value obtained for an amino-acid decarboxylase up to the present. The ornithine decarboxylase of *Bact. coli* has an optimum at *p*H 5·0 [Gale, 1940, 1].

l(−)-*Tyrosine decarboxylase.* Tyrosine is decarboxylated comparatively slowly by *Cl. aerofoetidum* at an optimum *p*H of 5·0 as shown by Fig. 3. Un-

fortunately it has been possible to obtain only one strain of this organism. The *p*H optimum for this one strain is the same as that found for *Strep. faecalis* [Gale, 1940, 2], but whereas that organism attacked only tyrosine, *Cl. aerofoetidum* also decarboxylates glutamic acid.

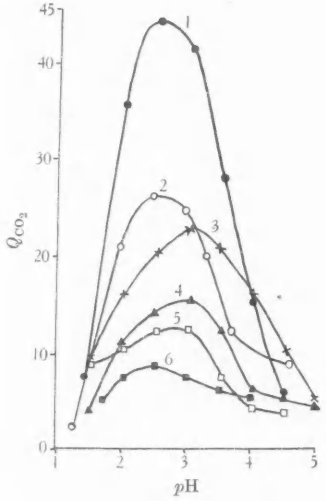

Fig. 1.

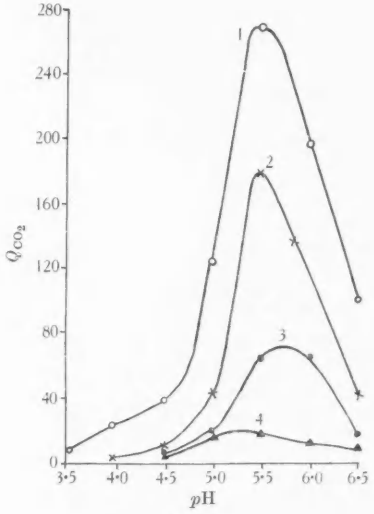

Fig. 2.

Fig. 1. Variation of histidine decarboxylase activity (Q_{CO_2}) with *p*H. 1, *Cl. welchii* B.W. 21. 2, *Cl. welchii* Chelsea. 3, *Cl. welchii* S. 107. 4, *Cl. welchii* S.R. 9. 5, *Cl. fallax* Morcom. 6, *Cl. bifermentans.*

Fig. 2. Variation of ornithine decarboxylase activity (Q_{CO_2}) with *p*H. 1, *Cl. septique* Pasteur III. 2, *Cl. septique* Carruthers. 3, *Cl. septique* Jerral. 4, *Pr. morganii.*

l(+)-*Glutamic acid decarboxylase.* Glutamic acid is attacked by all the strains of *Cl. welchii*, *Cl. bifermentans* and *Cl. aerofoetidum* tested and also by the majority of *Proteus* and coliform organisms. Fig. 4 gives the variation of the decarboxylase activity with *p*H in a selection of these cases; the *p*H optimum lies between 3·5 and 4·5, usually at 4·0. In all cases the *p*H-activity curve shows a rapid drop on the acid side of the optimum. In these studies of the decarboxylating powers of various bacteria, the glutamic acid enzyme has been the most frequently encountered and, in general, the most active of the amino-acid decarboxylases investigated. A quantitative study of the CO_2 liberated shows that only one of the —COOH groups of the glutamic acid molecule is removed and, as is shown below, the product has always proved to be γ-aminobutyric acid. It may be that γ-aminobutyric acid plays some important part in the cell metabolism.

Thermolability. In the original investigations with *Bact. coli* it was found that the activity of the washed suspensions fell off rapidly and that this loss was increased with rise of temperature. This thermolability affects all these investigations but it varies greatly from organism to organism and is not constant for any one enzyme; for instance, the glutamic acid decarboxylase of *Bact. coli* is found to be very sensitive to temperature, but the similar enzyme of *P. morganii* is far

less sensitive and, when working at the experimental temperature of 30°, a linear decarboxylation is often obtained lasting over an hour with this organism. Similarly the histidine decarboxylase of *Cl. fallax* Morcom maintains a steady rate at 30°, whereas in most other cases the rate of decarboxylation begins to fall off within 15 min. after mixing. For the experiments involving isolation of the product, quantities of washed suspension etc. were arranged so that the decarboxylation should be complete within an hour and such large scale experiments were checked manometrically.

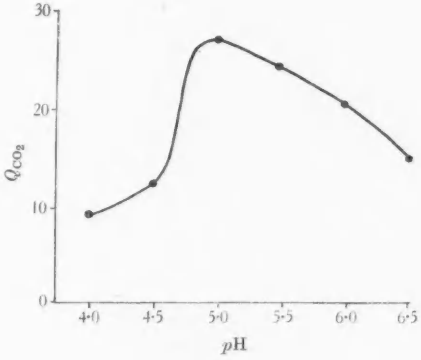

Fig. 3.

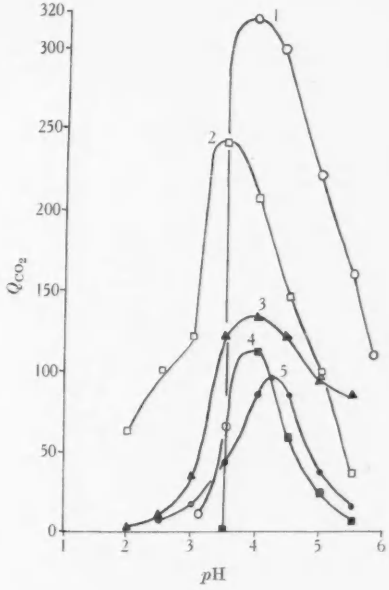

Fig. 4.

Fig. 3. Variation of tyrosine decarboxylase activity (Q_{CO_2}) with *p*H: *Cl. aerofoetidum.*

Fig. 4. Variation of glutamic acid decarboxylase activity (Q_{CO_2}) with *p*H. 1, *Cl. aerofoetidum.* 2, *Cl. welchii* Chelsea. 3, *Cl. welchii* B.W. 21. 4, *Pr. morganii.* 5, *Cl. bifermentans.*

Temperature coefficient. The temperature coefficient has been determined in most cases by carrying out manometric experiments under optimum *p*H conditions and at four different temperatures in each case. Fig. 5 shows the variation of initial Q_{CO_2} in the various cases with thermostat temperature. In most cases a straight line can be drawn through the points obtained for any one enzyme and organism, an exception being the ornithine decarboxylase of *Cl. septique* Pasteur which shows an 'optimum temperature' of 29–30°. There is a general falling-off of activity in most cases for temperatures above 40°. Glutamic acid decarboxylase of *Pr. morganii* shows an unusual variation with temperature, the Q_{CO_2} increasing very rapidly between 25 and 37° and then falling off almost to zero by 40°; only the early portion of this curve is shown on Fig. 5. Table 2 shows the temperature coefficients calculated from the lines shown in Fig. 5.

Effect of oxygen. With one exception the amino-acid decarboxylases of the *Clostridia* are unaffected by the presence of oxygen, the exception being the glutamic acid decarboxylase of *Cl. welchii*, a 50 % inhibition of which is obtained if air is used in the manometers instead of N_2.

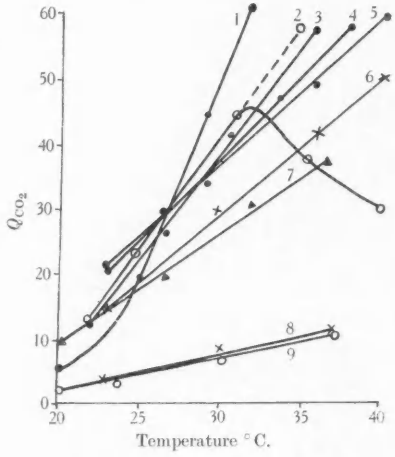

Fig. 5. Variation of activity with temperature. 1, *Pr. morganii*. 2, *Cl. septique* (reduced 5 ×). 3, *Cl. aerofoetidum* (reduced 10 ×). 4, *Cl. bifermentans*. 5, *Cl. welchii* B.W. 21. 6, *Cl. welchii* B.W. 21. 7, *Cl. aerofoetidum*. 8, *Cl. bifermentans*: *Cl. fallax*. 9, *Pr. morganii*.

•—• Glutamic acid. o—o Ornithine.
×—× Histidine. ▲—▲ Tyrosine.

Table 2. *Temperature coefficients of amino-acid decarboxylases*

Substrate	Organism	Temp. coeff.
Glutamic acid	*Cl. welchii* B.W. 21	1·9
	Cl. aerofoetidum 505	2·7
	Cl. bifermentans 2914	2·0
	Pr. morganii	4·3
Histidine	*Cl. welchii* B.W. 21	2·1
	Cl. bifermentans 2914	2·2
	Cl. fallax Morcom	2·1
Ornithine	*Cl. septique* Pasteur	2·45
	Pr. morganii	2·45
Tyrosine	*Cl. aerofoetidum* 505	2·1

Growth conditions necessary for the formation of the decarboxylases

Age of culture. 2 l. of the 2 % glucose broth medium were incubated in a flask fitted with a three-hole rubber bung: through the bung three glass tubes were passed, one connected to a hydrogen cylinder and conveying the gas below the level of the medium, a second reaching to the bottom of the flask and bent over outside to act as a sampling jet and the third a short tube connected outside to a Bunsen valve, the whole apparatus being assembled under sterile conditions. After inoculating the medium with the contents of a cultured meat-broth tube as usual, a stream of H_2 was swept through the flask to render the contents anaerobic.

Table 3. *Variation of decarboxylase activity with age of culture*

Age of culture hr.	Medium *p*H	mg. bact./ml. medium	Q_{CO_2} Histidine	Ornithine	Tyrosine	Glutamic acid
			Cl. welchii B.W. 21			
0	6·85	—	—	—	—	—
3	6·15	0·181	1·5	—	—	47
4	5·60	0·424	2·5	—	—	107
5	4·83	0·565	4·5	—	—	112
6	4·46	0·755	13	—	—	103
7	4·31	0·830	24	—	—	97
8	4·31	0·890	26	—	—	92
9	4·21	0·960	28	—	—	69
24	4·10	0·965	9	—	—	13
			Cl. septique Pasteur III			
0	7·59	—	—	—	—	—
2	7·40	0·030	—	167	—	—
3	7·23	0·081	—	474	—	—
4	6·92	0·134	—	251	—	—
5	6·59	0·185	—	194	—	—
6	6·37	0·226	—	150	—	—
7½	6·03	0·256	—	166	—	—
8½	5·90	0·270	—	201	—	—
10	5·82	0·282	—	183	—	—
26	5·53	0·311	—	159	—	—
			Cl. aerofoetidum 505			
0	7·23	—	—	—	—	—
3½	7·21	0·061	—	—	33	8·5
4½	6·92	0·122	—	—	30	7
5½	6·62	0·240	—	—	27	17
7	6·26	0·352	—	—	48	43
8	5·88	0·428	—	—	56	113
9	5·63	0·855	—	—	50	266
10	5·46	0·870	—	—	52	325
26	4·70	0·970	—	—	36	264
			Cl. bifermentans 2914			
0	7·04	—	—	—	—	—
3	6·35	0·168	2	—	—	12
4	5·98	0·396	3·5	—	—	92
5	5·79	0·528	7	—	—	125
6½	5·69	0·600	8	—	—	138
7½	5·64	0·660	10	—	—	132
8½	5·58	0·752	8	—	—	131
26	5·22	0·755	5	—	—	94
			Cl. fallax Morcom			
0	7·1	—	—	—	—	—
3	6·88	0·048	1	—	—	—
4	6·68	0·165	2	—	—	—
5	6·29	0·333	3·5	—	—	—
6½	5·77	0·480	5	—	—	—
7½	5·47	0·616	5	—	—	—
8½	5·32	0·720	6	—	—	—
9½	5·15	0·800	9	—	—	—
10½	5·12	0·780	12	—	—	—
26	4·63	0·765	6	—	—	—
			Pr. morganii			
0	7·19	—	—	—	—	—
2½	6·76	0·061	—	3	—	—
3½	6·55	0·146	—	8	—	—
4½	6·32	0·218	—	16	—	—
6½	5·81	0·346	—	12	—	—
7½	5·70	0·385	—	10	—	—
8½	5·67	0·400	—	7	—	—
26	5·19	0·400	—	6	—	—

In order to take samples at intervals the Bunsen valve was clipped off and the sampling jet opened, when samples of any desired volume could be removed aseptically by slightly increasing the H_2 pressure. In this manner the variation of the decarboxylase activities of the various organisms with 'age of culture' has been followed. The *Clostridia* grow extremely rapidly and turbidity is usually marked 2 hr. after inoculation so that sufficient organisms for the preparation of washed suspensions can be obtained from 300–400 ml. medium after 3 hr. Under these conditions active cell division ceases after 7–11 hr. depending on the organism used, *Cl. bifermentans* 2914 growing the most rapidly of all the organisms tested. With each sample the following determinations were made: *p*H, dry weight of organism present per ml. medium, and decarboxylase activity against the substrates concerned. The results obtained in representative cases are set out in Table 3. They show that, as usual, early cultures display little activity and that the decarboxylases appear at various stages in the growth cycle, reach maximum activity before growth ceases, and then fall off slowly in activity as the culture ages.

It is obvious that the appearance of the decarboxylases is not conditioned only by the age of the culture, as some enzymes, notably the histidine decarboxylase, appear much later in the growth period than others such as the ornithine decarboxylase. Fig. 6 shows the variation of the decarboxylase activity plotted not against the age of the culture but against the *p*H of the medium during growth. From this it appears that the enzymes develop as the *p*H of the medium approaches their optimum *p*H values. Thus it appears that the main factor in this variation of activity with age of culture is due to the variation in medium *p*H during growth. A point of possible theoretical interest is that the medium *p*H at which maximum activity is developed for each enzyme is approximately 1 *p*H unit to the alkaline side of the optimum *p*H for that enzyme. This seems to indicate that the actual *p*H in the vicinity of the enzyme surface within the actively fermenting organism may be below that measured in the medium as a whole.

The case of the ornithine decarboxylase of *Cl. septique* Pasteur is different from the others. A 2 hr. culture has little activity and there is a rapid increase of activity by the 3rd hr. followed by an almost equally rapid fall to a Q_{CO_2} of 150–180 at which level the activity stabilizes for 24 hr. Growth however continues for 8–9 hr. so that in this case there is a marked oscillation of activity during the early phase of growth and it appears on examination of stained films that the organism undergoes a structural change between 3 and 6 hr. The organisms obtained during the first 2·5 hr. stain homogeneously but as growth proceeds organisms appear which stain darkly at their ends and exhibit a granular structure, while spores are first seen at about the 4th hr. The fall in activity after 3 hr. is thus probably related to some structural change in the organism.

Effect of medium p*H during growth.* In the previous studies of amine production by bacteria [Gale, 1940, 1, 2] it has been shown that the amino-acid decarboxylases are produced by the organisms in response to an acid growth environment. That the same is true in the present studies has been indicated by Fig. 6. Flasks have been set up containing broth (without glucose) at adjusted *p*H values, inoculated with the organism, filled with H_2 and then incubated at 37° until growth reached a maximum. The *p*H of the media after growth was measured and the cultures made up into washed suspensions. It was very noticeable in the work with the *Clostridia* that whereas media adjusted before incubation to *p*H 8, 7 or 6 remained approximately at the adjusted values, those adjusted to values below 6 altered considerably during the growth of the

organism; thus a medium adjusted to *p*H 5·07 before incubation was found to have *p*H 5·91 on harvesting (*Cl. welchii* B.W. 21). This effect may be due to the greater formation and activity of the glutamic acid decarboxylase at low *p*H values. Fig. 7 shows the amino-acid decarboxylase activity of washed suspensions so prepared plotted against the average *p*H of the medium during growth. The *Clostridia* do not grow very well below *p*H 5·5 in the absence of carbohydrate. The results show, as before, that the enzymes are formed in response to an acid growth environment. In many cases, also as before, the organism growing at *p*H 5 shows as much or greater activity than when grown in glucose broth, showing that the function of the glucose in promoting amine production is to act as a source of fermentation acid. An apparent exception is the histidine decarboxylase of *Cl. welchii*, *Cl. fallax* and *Cl. bifermentans*. In the case of the histidine decarboxylase of *Bact. coli* [Gale, 1940, 1] the enzyme, having an

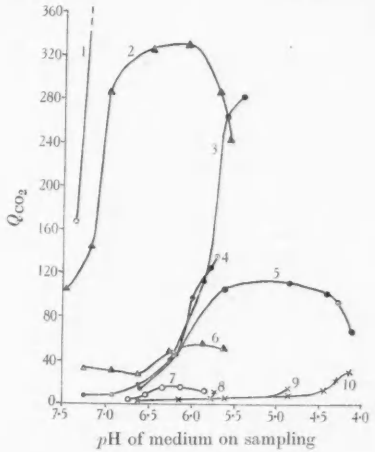

Fig. 6.

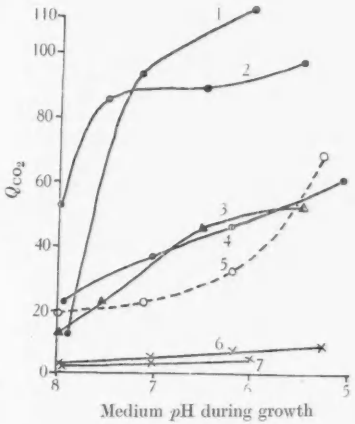

Fig. 7.

Fig. 6. Variation of decarboxylase activity with medium *p*H during growth in glucose broth. 1, *Cl. septique*. 2, *Strep. faecalis* [Gale, 1940, 2]. 3 and 6, *Cl. aerofoetidum*. 4 and 8, *Cl. bifermentans*. 5 and 10, *Cl. welchii* B.W. 21. 7, *Pr. morganii*. 9, *Cl. fallax*.

Fig. 7. Effect of growth *p*H on decarboxylase activity of washed suspensions. 1 and 7, *Cl. bifermentans*. 2 and 3, *Cl. aerofoetidum*. 4 and 6, *Cl. welchii* B.W. 21. 5, *Cl. septique* (reduced 10×).

•—• Glutamic acid. o—o Ornithine.
×—× Histidine. ▲—▲ Tyrosine.

optimum *p*H at 4·0, behaved in this respect like the other decarboxylases, but as a result of the lower *p*H optimum of the *Cl. welchii* histidine decarboxylase, the enzyme in this case requires more acid conditions for its formation than are necessary with *Bact. coli*. Thus in glucose broth the histidine enzyme only appears late in the growth period when the medium *p*H has fallen below 5 (see Fig. 6). When *Cl. welchii* is grown in non-carbohydrate media it is found that the lower the growth *p*H, the more active the decarboxylase, but the activity of a culture grown at *p*H 5 has $Q_{CO_2} = 10$ compared with a value of 60–70 for the same strain

(B.W. 21) grown in the presence of glucose. It is not possible to obtain cultures from non-carbohydrate media at *p*H values much less than 5 so that it appears either that glucose plays a definite part in promoting the formation of this particular enzyme, or that a strongly saccharolytic organism like *Cl. welchii* is able to produce a *p*H in its immediate vicinity during fermentation below that of the medium in general. Whatever may be the reason, it is true to say that *Cl. welchii* will not form the histidine decarboxylase or produce histamine to any extent *in vivo* unless it is actively fermenting carbohydrate.

As has been noted in the case of the ornithine decarboxylase of *Bact coli*, the ornithine enzyme of *Cl. septique* shows a very marked response to acid growth conditions; the ornithine decarboxylase activity is scaled down 10 times in Fig. 7 in order to compare with the other curves.

Growth temperature. The decarboxylases of *Bact. coli* are formed more actively when the organisms are grown at 27° than at 37°. This does not seem to be the case with either the *Clostridia* or *Proteus*, growth temperature having little effect in most cases while the histidine enzyme is better formed at the higher temperature as, presumably, fermentation occurs more rapidly under such a growth condition.

Isolation and identification of the amines produced

The products of the decarboxylation processes have been isolated in every case from large-scale experiments based on and controlled by manometric work. The manometric experiments indicate in every case that the decarboxylation under optimum conditions proceeds to completion since the theoretical volume of CO_2 is liberated within experimental error. In most cases the entire organisms obtained from 800 ml. glucose broth have been used for each isolation and the concentration of the washed suspension, together with the quantities of buffer and amino-acid, have been arranged so that the decarboxylation should go to completion within 1 hr. The full details of the experimental procedure and isolation are the same as previously described [Gale, 1940, 1, 2]. The yields on isolation of the products of decarboxylation usually amount to 60–70 % of theory. Analyses are by Weiler.

Histidine decarboxylase: product histamine isolated as dipicrate. *Cl. welchii* B.W. 21. Quantities: 20 ml. washed suspension, 15 mg./ml.; 20 ml. 0·1 *M* phthalate buffer *p*H 2·5; 20 ml. *M*/60 *l*(−)-histidine monohydrochloride (neutral). Yield 135 mg.—70 % theory. M.P. 239° (corr.). (Found: C, 35·95; H, 2·67; N, 22·25 %. Calc.: C, 35·97; H, 2·64; N, 22·2 %.)

Similar experiments were carried out with:

Cl. welchii S.R. 9 (*p*H 3·0). (Found: N, 22·08 %.) M.P. 238°. Yield 63 %.

Cl. welchii Chelsea (*p*H 2·5). (Found: N, 22·00 %.) M.P. 241°. Yield 58 %.

Cl. bifermentans 2914 (*p*H 2·5). (Found: C, 36·1; H, 2·68; N, 22·05 %.) M.P. 240°. Yield, 83 %.

Cl. fallax Morcom (*p*H 2·5). (Found: C, 36·2; H, 2·62; N, 21·9 %.) M.P. 240°. Yield, 68 %.

Tyrosine decarboxylase: product tyramine isolated as dibenzoyl deriv. *Cl. aerofoetidum* 505. Quantities: 15 ml. washed suspension 20 mg./ml.; 15 ml. 0·1 *M* phthalate buffer *p*H 5·0; 15 ml. *M*/60 *l*(−)-tyrosine. Yield 65 mg.—74 % theory. M.P. 171°. (Found: C, 76·10; H, 5·47; N, 3·89 %. Calc.: C, 76·50; H, 5·54; N, 4·06 %.)

Ornithine decarboxylase: product putrescine isolated as dipicrate. *Cl. septique* Pasteur III. Quantities: 30 ml. washed suspension 6 mg./ml.; 30 ml. 0·01 *M* phosphate buffer *p*H 5·5; 30 ml. *M*/60 *l*(+)-ornithine dihydrochloride (neutral).

Yield 197 mg.—72 % theory. Decomposes at 250°. (Found: C, 35·69; H, 3·67; N, 20·00 %. Calc.: C, 35·03; H, 3·65; N, 20·44 %.)

Glutamic acid decarboxylase: product γ-aminobutyric acid. Neuberger [1937] states that the dissociation constants of γ-aminobutyric acid are $pK_1 = 4{\cdot}23$ and $pK_2 = 10{\cdot}43$ whereas the author has shown [1940, 1] that the dissociation constants for the α-aminobutyric acid are very different from these, particularly pK_1 which lies in the region of 2. Accordingly it is a simple matter to distinguish between the two possible products of the glutamic acid decarboxylation by carrying out a titration curve on the experimental material and so determining the value of pK_1. The complete isolation has been carried out in the case of *Bact. coli*.

Found: *Cl. welchii* B.W. 21 (*p*H 4·0). $pK_1 = 4{\cdot}15$.
Cl. aerofoetidum 505 (*p*H 4·0). $pK_1 = 4{\cdot}25$.
Cl. bifermentans 2914 (*p*H 4·0). $pK_1 = 4{\cdot}30$.
Pr. morganii (*p*H 4·0). $pK_1 = 4{\cdot}25$.

Thus the product in all cases of glutamic acid decarboxylation studied has proved to be the γ-aminobutyric acid.

The production of histamine by Cl. welchii in vivo

It has been shown that whenever *Cl. welchii* grows in the presence of fermentable carbohydrate, conditions are such that histamine is produced from free histidine. *Cl. welchii* ferments muscle glycogen with the production of acid and gas almost as rapidly as it attacks glucose, the glycogen fermentation being strictly adaptive. *Cl. welchii* B.W. 21 grown in the presence of glycogen possesses a histidine decarboxylase Q_{CO_2} of 30–50 determined under the usual conditions. Thus it seems probable that when *Cl. welchii* invades muscle tissue conditions arise which are favourable for the production of histamine, as the fermentation of muscle glycogen would produce the necessary low *p*H, particularly in 'pockets' in such a heterogeneous medium, and the proteolytic activities of the organism would liberate histidine. It seems unlikely that sufficient histamine would be formed under these conditions to produce any symptoms unless in mixed or massive gangrene infections. This aspect of the investigation is dealt with in the Addendum to this paper. Since *Cl. paludis* and the Lamb dysentery organism behave as other *welchii* strains in producing histamine, this may play a part in the actiology of diarrhoea in lambs.

Inhibitors of histamine production by Cl. welchii

The production of histamine by *Cl. welchii* can be checked either by inhibition of histidine decarboxylase or by inhibition of fermentation during growth. Of a range of substances tested on washed suspensions, $KMnO_4$ and chloramine T prove to be the most active inhibitors, *M*/1000 solutions of either completely inhibiting the histidine decarboxylase and the fermentation of either glucose or glycogen under optimal conditions. *M*/1000 iodoacetate inhibits fermentation reactions but has no effect on histidine decarboxylase. Acriflavin, though very effective as a disinfecting agent as far as *Cl. welchii* is concerned, does not affect the enzymes in question at concentrations less than *M*/10. The decarboxylase is unaffected by concentrations less than *M*/100 of the following: cyanide, urethane, urea, salicylic acid, fluoride, sulphanilic acid, hydrazine, H_2O_2, etc., or by bubbling with O_2.

Discussion

The work presented above gives a general idea of the decarboxylating powers of the *Clostridium* and *Proteus* organisms and the conditions under which such activities can be expected to function. It cannot be regarded as a comprehensive survey as it may well be that some strains of the organisms reported as not showing decarboxylating activities will possess such enzymes, just as only one strain of *Cl. fallax* tested can produce histamine. Those members of the *Clostridium* group which do possess decarboxylases do not appear to have any other marked property in common that distinguishes them from the remainder; they are all strongly saccharolytic, but others of the most saccharolytic of the group, e.g. *Cl. butyricum*, are inactive as far as can be decided at present. *Cl. welchii*, *Cl. septique*, *Cl. bifermentans* and *Cl. aerofoetidum* are all classified in the saccharolytic and proteolytic group of the M.R.C. classification whereas *Cl. fallax* is stated to possess no proteolytic activity. Strains of all these organisms have been reported in the contents of the intestine. Of 9 strains of *Proteus* organisms tested, 7 attacked glutamic acid and 3 ornithine so that the amine-producing activities of this group do not appear to be marked.

Summary

1. Washed suspensions have been prepared of organisms from the groups *Clostridium* and *Proteus* grown in a 2% glucose casein-digest broth and these have been tested for their power to decarboxylate amino-acids.

2. *l*(−)-Histidine is decarboxylated to histamine by strains of *Cl. welchii*, *Cl. bifermentans* and *Cl. fallax*; *l*(−)-tyrosine is decarboxylated to tyramine by *Cl. aerofoetidum*; *l*(+)-ornithine is decarboxylated to putrescine by *Cl. septique* and *l*(+)-glutamic acid is decarboxylated to γ-aminobutyric acid by *Cl. welchii*; *Cl. aerofoetidum* and *Cl. bifermentans*. Of 9 strains of *Proteus* organisms tested, 7 decarboxylated glutamic acid and 3 ornithine.

3. The *p*H optimum for histidine decarboxylase lies between 2·5 and 3·0; for ornithine decarboxylase at 5·5; for tyrosine decarboxylase at 5·0 and for glutamic acid decarboxylase at 4·0.

4. The enzymes appear to vary in thermolability from organism to organism but all have temperature coefficients of the order of 2.

5. When the organisms are grown in glucose broth, early cultures have little activity, the decarboxylases developing later in the growth period as the *p*H of the medium approaches their optimum *p*H. The ornithine decarboxylase of *Cl. septique* is exceptional in that its activity reaches a maximum and then falls again within the first 3 hr. of growth; the loss of activity before growth ceases appears to be associated with a change in structure of the organism.

6. In the absence of carbohydrate the decarboxylase activity of the organism depends upon the *p*H of the medium during growth, low growth *p*H promoting high decarboxylase activity. The histidine decarboxylase activity of *Cl. welchii* is considerably higher when the organism is grown in the presence of fermentable carbohydrate then when it is grown in a non-carbohydrate broth at *p*H 5.

7. The possible pathogenic significance of histamine production by *Cl. welchii* is discussed and methods for its prevention noted.

The author wishes to thank Dr M. Stephenson and Prof. Sir Frederick Hopkins for their interest and encouragement throughout the course of this work. He is indebted to the Royal Commissioners for the Exhibition of 1851 for the Senior Studentship during the tenure of which this work has been carried out.

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ADDENDUM

Plasma histamine in gas gangrene

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If the symptoms of *Cl. welchii* infection are in part due to histamine intoxication, the histamine concentration of the plasma should be significantly increased. Information about changes in the histamine content of the whole blood is of doubtful value, since, as Code [1937] has shown, most of the blood histamine is in the formed elements. Cat's arterial blood, treated with heparin and centrifuged without delay, yields a plasma whose histamine equivalent may be determined directly upon the atropinized guinea-pig's ileum. Normal cat's plasma, obtained under 'dial' anaesthesia, has a histamine equivalent of not more than 0·01 μg./ml. We have observed that the histamine equivalent of the plasma may be raised to 0·45 μg./ml. by the intravenous injection of 0·5 mg. of histamine per kg. of body weight, or to 0·04 μg./ml. by the subcutaneous injection of the same dose. No obvious symptoms are produced in a cat given 0·5 mg./kg. of histamine subcutaneously.

We have infected cats with a histamine-producing strain of *Cl. welchii* provided by Dr Gale. They were anaesthetized with 'dial', and were maintained under the anaesthesia throughout the experiment. They received an injection into the soleus or gastrocnemius muscle of one leg of 2–6 ml. of a meat-broth culture aged 15–20 hr., and the plasma was examined 2–6 days later. In those animals in which the infection became fully developed, the limb was swollen and crepitant, and the skin covering it discoloured: the muscles of the calf immediately surrounding the site of injection were broken down to a pulpy mass, and in certain instances there was a considerable collection of thick creamy pus which yielded only *Cl. welchii* on culture. Some of the animals died, but the cat appears able to withstand an extensive involvement of one limb without succumbing to the effects of toxaemia. In no instance could *Cl. welchii* be recovered from the blood.

The histamine equivalent of the plasma from the infected animals has in no case been greater than 0·02 μg./ml., and in two animals in which the infection was advanced and the assay particularly satisfactory, values of 0·008 and 0·007 μg./ml. were obtained. We took the occasion, in four animals, to determine the histamine equivalent of normal and infected muscles. The histamine equivalent of the infected muscles was greater, by 30 to 230 %. If the normal histamine content of cat's muscle is taken to be 1 μg./g., it can be calculated that at least 300 g. of infected tissue would be required to yield 1 mg. of histamine. The histamine equivalent of the purulent exudate in one animal was about the same as that of normal muscle.

Clinical experience shows, and we have found in the cat, that an infection with *Cl. welchii* proceeds more rapidly if other anaerobes such as *Cl. sporogenes* or *Cl. histolyticum* are present. These organisms, although not themselves histamine producers, are actively proteolytic, and might be expected, by increasing the amount of histidine available, to accelerate the liberation of histamine by *Cl. welchii*. In a cat which had received a mixture of 20 hr. broth cultures of *Cl. welchii* (1 ml.) and *Cl. histolyticum* (0·5 ml.), the infection was well developed in 28 hr., involving the thigh muscles as well as the gastrocnemius. The histamine equivalent of the plasma was less than 0·005 μg./ml. The histamine equivalent of the infected and pultaceous muscle was about 30 % higher than that of the normal side.

It would appear, therefore, that in cats infected with *Cl. welchii*, no significant pathological effect is produced by the absorption of histamine into the blood stream from the infected tissue.

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II. THE COLORIMETRIC ESTIMATION OF URIC ACID IN FOWL'S EXCRETA

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NUMEROUS methods have been elaborated for the estimation of uric acid in avian excreta. These methods and their defects have been admirably reviewed by Fritz [1935]. In this laboratory a modification of Woodman's [1924] method has been in use, in which the nitrogen in the precipitate obtained is estimated by the Kjeldahl method. It is found that this nitrogen is about 35% less than would correspond to the weight of the precipitate if the latter were pure uric acid. It has been assumed that the impurities present in the precipitate contain no nitrogen and if this is the case the method may be accurate, but if nitrogenous impurities are present the results must be too high. Fritz [1935] has found that uric acid is liable to be destroyed by extraction with lithia, and Folin & Denis [1912] have called attention to the fact that uric acid is very rapidly destroyed on boiling in alkaline solution. The results may be too low on this account. In addition, the method is very laborious, owing to the filtration difficulties and the numerous processes involved. Consequently it is not very practical for metabolic work in which it is necessary to make a large number of estimations and to obtain the results quickly.

St John & Johnson [1931] have worked out a more refined isolation technique in which piperidine replaces lithia as the solvent for uric acid. This method appears to be reliable, but from the results published in their paper it seems that the quantity of nitrogen found in their precipitate averages 2·7% less than corresponds with the weight of precipitate. There is thus still a small error caused by lack of specificity. If there is any nitrogenous impurity in the precipitate the error may be greater than 2·7%. If on the other hand the uric acid content is calculated from a nitrogen estimation on the precipitate to eliminate the error, the method becomes nearly as cumbrous as Woodman's procedure, and in any case a more rapid method is desirable. Fritz [1935] also states that loss of uric acid is very liable to occur in piperidine solution when this method is employed.

The author has tried to separate uric acid added to human faeces by the methods of St John & Johnson [1931] and of Woodman [1924]. It was not possible to get a full recovery, because the filtration processes under these conditions are very lengthy and loss of uric acid occurred in alkaline solution, but it was found that by St John & Johnson's method the nitrogen obtained from the precipitate was 21% less than corresponded to the weight of precipitate, while by Woodman's method it was 34% less. There was no evidence of nitrogenous impurities in the precipitate obtained by St John & Johnson's method, but nitrogenous impurities were present in the precipitate obtained by Woodman's method.

The colorimetric method is much less laborious than the isolation methods, and it has been developed to a high degree of specificity for estimations in blood and urine, chiefly by Benedict and by Folin. Preliminary experiments were

made by the author, using the Benedict & Franke [1922] method for urine, on phosphate and lithia extracts of dried sheep's and human faeces to which 7 % of uric acid had been added. The uric acid recovered exceeded by 4·3 % the amount added to sheep's faeces and by 3·3 % in the case of human faeces. The extracts were not sufficiently dark coloured to affect the colorimeter readings. It was clear from these experiments that the colorimetric method was highly specific for uric acid in faecal extracts and that it would only be necessary to introduce one stage of purification to obtain an accurate method.

The method described in this paper has been worked out for use with Benedict's [1922] arsenophosphotungstic acid reagent and Newton's [1937] lithium arsenotungstate reagent. A great deal of work has been published on methods of intensifying the blue colour. Christman & Ravwitch [1932] found no true proportionality of colour intensity to the concentration of uric acid when Benedict's [1922] 5 % cyanide solution was used. Moreover, it varied with different samples of cyanide and also was less reliable for diluted urine than for pure uric acid solutions. They found that they could correct the proportionality by adding 10 % of sodium carbonate to the cyanide solution and claimed that this solution gave a higher degree of specificity to the colour reaction. They considered that the variation in different samples of cyanide was due to their different contents of carbonate. In the author's experience however the proportionality is not the same with different samples of cyanide of similar origin used for making up Christman's solution, so that it seems that some other factor besides the carbonate content is involved. The author has compared the proportionality observed when Christman's solution was used with that observed when Folin's [1933] cyanide urea solution (which is free from carbonate) was employed. Both solutions were prepared from the same sample of sodium cyanide (B.D.H. pure). Uric acid standards containing 0·2, 0·3 and 0·4 mg. per 10 ml. were prepared by dissolving pure uric acid in warm lithium carbonate and adding acid lithium chloride, as described later, to prevent turbidity from developing during the colour reaction. 10 ml. of the standard solution, 5 ml. of cyanide and 2 ml. of undiluted Newton's reagent were used to develop the colour. On comparing the 0·2 and 0·4 mg. standards with the normal standard of 0·3 mg., the proportional colour intensity was found to increase about 5 % over this range as the concentration of uric acid was increased when Christman's solution was used. That is, the 0·4 mg. standard was 2·5 % too high and the 0·2 mg. standard was 2·5 % too low. When Folin's cyanide urea solution was employed the proportional colour intensity decreased 6 %. Thus the effect of adding the carbonate was to reverse the error in proportionality. It decreased the total intensity of the colour as Folin states. It seems that there should be an optimum quantity of carbonate which will give true proportionality with any given sample of cyanide, though this quantity varies with different samples. For example, a second sample of cyanide (also B.D.H. pure) gave true proportionality with Newton's reagent and Christman's cyanide. With a third sample (B.D.H pure) the proportional colour intensity increased 5·5 %, practically the same amount as with the first sample.

Similar results were obtained with 2 ml. of the Benedict & Franke [1922] reagent. With the first sample of cyanide the proportional colour intensity increased 7 %, the second gave true proportionality, while the third gave an increase of 5 %.

A large number of experiments on proportionality were carried out with extracts of fowl's excreta both by the direct and indirect methods described later. In contrast to the experience of Christman & Ravwitch [1932] with diluted

human urine, the proportionality was found to be exactly the same with extracts of excreta as with lithium carbonate solutions of uric acid.

When an ammoniacal precipitating solution is used for the uric acid, the residual ammonia remaining in the precipitate can depress the colour reaction. This depressant effect is considerably greater for cyanide solutions with high driving power, such as Benedict's [1922] and Folin's [1933], than for Christman's solution. When the Benedict & Hitchcock [1915] 'ammoniacal silver magnesium precipitating solution' is used, as in the method described below, there appears to be an advantage in using Christman's solution for the colour development. Moreover, Christman & Ravwitch [1932] claim that their solution is more specific for uric acid than a 5 % solution of sodium cyanide without the addition of carbonate. Folin [1934], on the other hand, considers that increasing the driving power by removal of traces of carbonate increases the specificity and therefore uses cyanide solutions in which the carbonate is completely removed. For the estimation of uric acid in fowl's excreta no marked difference has been observed as regards the specificity of Folin's and Christman's solutions, but if ammonia can affect the action of Folin's solution more than that of Christman's solution, it may be that other extraneous factors can also affect it more than Christman's solution under practical conditions. For this work therefore Christman's solution has been used as the standard reagent. It has been confirmed that its driving power remains unimpaired for several months.

The preliminary experiments reported above on faecal extracts showed that to obtain the necessary specificity it was desirable to separate the uric acid from the extract prior to developing the colour. For the precipitation of uric acid the zinc hydroxide method of Morris & Macleod [1922] and the Salkowski reaction as employed by Benedict & Hitchcock [1915] have been tried. The zinc method appeared to bring down a considerable quantity of colouring matter as well as the uric acid. Moreover, the zinc was found to exert a depressant effect on the colour reaction, as Kern & Stransky [1937] had also observed, so that only 97 % recovery could be obtained from lithium carbonate solutions of uric acid. The results by this method, after correcting for the loss in the recovery, were practically the same as with the Benedict & Hitchcock [1915] method, in spite of the lack of specificity. Benedict and his co-workers consider that the Salkowski reaction provides the most specific method of precipitating uric acid, and it has therefore been adopted as the standard method. Here again difficulty was experienced in getting a full recovery of uric acid from lithium carbonate solution. This is partly because the reaction is not absolutely quantitative [Folin & Wu, 1919], and partly because the residual ammonia present in the precipitate can depress the colour reaction. The best results were obtained by using the proportions of precipitant recommended by Benedict & Hitchcock [1915], namely, 1 ml. precipitant to 5 ml. extract, and not 5 ml. precipitant to 5 ml. extract as used by Christman & Ravwitch [1932]. If the neck of the centrifuge tube is swabbed out carefully after draining and a current of air is passed over the precipitate, most of the residual ammonia can be eliminated so as not to interfere with the colour reaction. By this means a recovery of 98·5 % was obtained and the results were corrected accordingly. This correction may vary up to 1 % with individual workers and should be determined individually. The amount of ammonia remaining in the silver magnesium urate precipitate after passing the current of air should not exceed 1 ml. $N/10$.

To decompose the precipitate and remove the silver, which may interfere with the colour reaction, 7 % lithium chloride in 0·125 N HCl was used in place of Folin's acid NaCl procedure. The advantage in the use of lithium chloride is that

it prevents the development of turbidity during the colour reaction. It was found that if lithium chloride was not present the urea in the cyanide solution was barely sufficient to prevent turbidity when Benedict's reagent was used, and definitely insufficient with Newton's reagent. Benedict & Behre [1931] have also used an acid lithium chloride solution for this reason. The decomposition of the precipitate by acid chloride not only removes the silver, but has the advantage of bringing down most of the colouring matter which may be present, so that a colourless solution of uric acid is obtained even from the most deeply coloured extracts of excreta. It is found that no uric acid is lost by this procedure, even though the concentration of uric acid approximates to, or in some cases even exceeds, its solubility in acid solution.

The author, like Christman, has not found Benedict & Hitchcock's [1915] phosphate standard uric acid solution to be reliable. Folin's [1930] most recent formalin standard has proved to have excellent keeping qualities. On dilution it was found to be exactly equivalent in colour-producing property to a lithium carbonate solution of uric acid of the same strength. A diluted solution of this standard containing 0·3 mg. uric acid in 10 ml. is prepared for use and this will keep for 14 days. A quantity of acid lithium chloride solution equal to that present in the unknown must be added to the standard, because chloride intensifies the colour reaction, at all events when Benedict's arsenophosphotungstic acid reagent is employed.

Fritz [1935] found that loss of uric acid is very liable to occur when it is extracted from the excreta by lithia or piperidine. The author has found that piperidine depresses the colour reaction. Phosphate is not suitable because it interferes with the precipitation of uric acid, causing turbidity in the subsequent colour reaction, though it gives only a lightly coloured extract. Dilute lithium carbonate has been successfully employed by Yamaguchi [1930] and by Fürth & Edel [1933] for the extraction of uric acid from tissues. It has proved very successful for extracting the uric acid from fowl's excreta in the method described below.

Woodman [1924] considered it desirable to convert any ammonium urate present in the excreta into uric acid with HCl before extracting with lithia, since unless this were done the extraction of uric acid was apparently not complete. St John & Johnson [1931] also adopt this procedure before their piperidine extraction. Some experiments were made to find out whether this preliminary treatment was necessary in the case of fowl's excreta. Samples were estimated by the standard colorimetric technique described below, with and without pretreatment with HCl. 0·8 g. of excreta was covered with 7 ml. of distilled water and 7 ml. 0·3 *N* HCl were added. The mixture was brought to boiling point and held there for 4 min. In the first two experiments enough lithium carbonate was added to neutralize the HCl in addition to the 12 ml. normally used for the extraction. In the other two experiments the mixture was left for 24 hr. after boiling with HCl and then filtered and washed once with distilled water. The residual HCl was neutralized with lithium carbonate and extracted in the same way. The results of these experiments were as follows (Table 1).

Table 1

	% uric acid on air-dried wt.	
No. of exp.	With HCl pretreatment	Without HCl pretreatment
1	8·49	8·43
2	8·57	8·60
3	10·13	10·32
4	9·25	9·10

It is apparent that preliminary treatment with HCl is unnecessary.

The following standard procedure has been adopted for the estimation of uric acid in avian excreta. Since eight estimations can be performed in one day, there is a considerable saving of labour as compared with the older methods.

Method

1. Enough air-dried excreta to give 50–100 mg. uric acid is weighed and washed into a 250 ml. volumetric flask with 20 ml. of distilled water.

2. 12 ml. 0·5 % lithium carbonate solution or, if more than 0·8 g. excreta were taken, 15 ml., are added. The flask is heated on a water bath for 15 min., cooled immediately and made up to volume.

3. The extract is filtered, the first few ml. being rejected if there is any turbidity.

4. 5 ml. filtered extract are pipetted into a 25 ml. centrifuge tube and 1 ml. of the Benedict & Hitchcock [1915] 'ammoniacal silver magnesium precipitant' is added. The liquid is stirred very carefully with a fine glass rod so that it does not splash up the sides of the tube and is then centrifuged for 5 min.

5. The supernatant liquid is poured off and the tube is drained for 2 min. Its neck is then swabbed with a filter paper. The tube is placed in a beaker of hot water (70–80°) and a current of air is passed for 1 min. over the precipitate to remove ammonia. The precipitate is dislodged from the bottom of the tube by a smart tap, the tube is replaced in the beaker of hot water and the current of air is passed for another minute.

6. 20 ml. 7 % lithium chloride solution in 0·125 *N* HCl are added and the contents of the tube are stirred very thoroughly with a fine glass rod until all the precipitate is decomposed. The tube is then centrifuged for 2–3 min.

7. The supernatant liquid is poured into a 50 ml. volumetric flask, 5 ml. of the acid lithium chloride solution are added to the centrifuge tube and 10 ml. distilled water. The contents are stirred and centrifuged again for 2–3 min.

8. The supernatant liquid is poured off into the 50 ml. flask and the liquid is made up to volume with distilled water.

9. The standard and unknown are prepared simultaneously for the colour development. 10 ml. of the solution in the 50 ml. flask and of the diluted standard are pipetted each into 50 ml. volumetric flasks. 5 ml. of the Christman's cyanide solution are added and either 2 ml. arsenophosphotungstic acid reagent or 1·5 ml. Newton's lithium arsenotungstate reagent undiluted; the flasks are shaken gently. The colour is allowed to develop for 5 min. with Benedict's reagent or 8 min. with Newton's reagent, and the mixtures are made up to volume with distilled water.

10. The colours are compared in a colorimeter immediately. Readings between 15 and 30 mm. are satisfactory provided that the reagents are adjusted to give true proportionality as described above. The results are corrected for the empirical loss in the recovery of uric acid after precipitation.

Recovery of uric acid from fowl's excreta

The following experiment was performed to test the recovery of different quantities of uric acid from fowl's excreta. A sample of excreta was separated mechanically as far as possible from uric acid and dried. Different quantities of uric acid were added to this, and the uric acid was estimated by the standard colorimetric method described above, using Benedict's colorimetric reagent

The results are given in Fig. 1. The amounts of uric acid added were recovered quantitatively over the whole range of conditions likely to be encountered in practice, from the low uric acid content of excreta from birds on a nitrogen-free ration to the high content which is obtained when they are on a nitrogen-rich ration. It appears that the amount of protein which is present in the excreta is

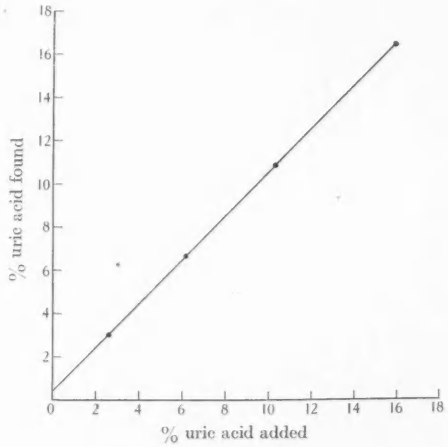

Fig. 1. Recovery of uric acid added to 'nearly uric acid-free' fowl's excreta.

not sufficient to affect the precipitation at low concentrations of uric acid. The graph (Fig. 1) does not run through the origin because there was a small quantity of residual uric acid in the excreta after the mechanical separation.

Specificity

To test the specificity of the method it was necessary to add the uric acid to sheep's and human faeces because it was impossible to obtain fowl's excreta completely free from uric acid. A lithium carbonate extract was prepared from dried sheep's faeces, and a uric acid solution in lithium carbonate was added to this to provide the equivalent of 7·5% of uric acid reckoned on the air-dried weight of the faeces. Thus, any error in the result could not be caused by failure of the extraction process, but must be due to lack of specificity. The uric acid content of this mixture was then estimated by the standard method, using Benedict's colorimetric reagent. The results were as follows (Table 2):

Table 2

No. of exp.	% uric acid added	% uric acid recovered
1	7·50	7·55
2	7·50	7·58
3	7·50	7·58
4	7·50	7·53
Mean	7·50	7·56

Further experiments were performed on sheep's and human faeces. Ammonium urate instead of uric acid was added before extraction and Newton's colorimetric reagent was used. The results are given in Table 3.

Table 3

Source of faeces	Weight of air-dried faeces g.	Ammonium urate added g.	Ammonium urate recovered g.
Sheep	0·700	0·0805	0·0810
,,	0·748	0·0825	0·0800
,,	1·331	0·0835	0·0801
,,	1·400	0·0815	0·0829
,,	1·403	0·0830	0·0840
Human	0·754	0·0825	0·0833
,,	1·503	0·0910	0·0946

These tests show that the method is highly specific and gives good results under conditions which caused a failure of the methods of Woodman [1924] and of St John & Johnson [1931] (see p. 81).

Experiments were also carried out to test the specificity of the Benedict and Newton colour reagents in the presence of certain other purine bodies which are also precipitated by the ammoniacal silver magnesium precipitant. Solutions or suspensions were made up in lithium carbonate of guanine, adenine, hypoxanthine and xanthine containing 0·75 mg. of the purine bodies and 0·3 mg. of uric acid per 10 ml. of solution. The colours produced by 10 ml. of these solutions were compared with that produced by 10 ml. of a standard uric acid solution (0·3 mg. per 10 ml.). Adenine and hypoxanthine gave no colour with either reagent. Xanthine gave 1/40th of the colour which would be given by the same quantity of uric acid when Newton's reagent was used for the colour reaction, and 1/30th of the colour with Benedict's reagent. Guanine gave 1/70th of the colour with Newton's reagent and 1/25th of the colour with Benedict's reagent. Thus the small quantity of the other purine bodies precipitated with uric acid could not affect the results. The Newton reagent appears to be rather more specific than the Benedict reagent, as Newton states. Fürth & Edel [1933], using Folin's colorimetric reagent, have also found that the decomposition products of the nucleic acids are either not precipitated or give no colour.

The possibility of using a direct method for the estimation of uric acid in avian excreta has been examined. Estimations made directly on the lithium carbonate extracts of the excreta have been compared with those made on the same extracts by the standard indirect method described above. Both Newton's and Benedict's colorimetric reagents were used in these determinations, and some experiments were made using Folin's carbonate-free cyanide urea solution. For the direct estimations with Christman's cyanide solution the lithium carbonate extracts were diluted 5 times to give a convenient concentration of uric acid, and the same amount of acid lithium chloride was added as was contained in the standard. For the direct estimations with Folin's cyanide-urea solution a standard was prepared containing 0·06 mg. uric acid per 10 ml. and the extracts of the excreta were diluted 25 times. To develop the colour when Folin's cyanide solution was used, 10 ml. diluted extract were taken, 5 ml. cyanide-urea solution and 1 ml. undiluted Newton's reagent were added. The volume was made up to 20 ml. after 10 min. The results of these experiments are given in Table 4.

The specificity of Newton's reagent combined with Christman's cyanide solution is high when used directly on the lithium carbonate extract. The results

obtained by this method were on the average 1·8 % higher than those obtained by the indirect method. The Benedict reagent was not quite so specific, yielding results which averaged 3·9 % above those obtained by the indirect method. The experiments in which the Newton reagent was combined with Folin's cyanide-urea solution gave values which were intermediate between those of the other two direct methods, being 2·6 % above those of the indirect method. Samples 5, 6, 7 and 8 were used for comparing the Newton and the Benedict reagents in the indirect method in conjunction with Christman's solution. The results for these samples are not the same for the two series, because they were part of a series of estimations carried out at the extremes of the range of uric acid concentrations to test the proportionality of the colour to the uric acid concentration.

Table 4

	Direct method		Indirect method	
No. of sample	% uric acid on air-dried wt.	Colorimetric technique	% uric acid on air-dried wt.	Colorimetric technique
1	12·68	N C	12·51	N C
2	11·21	N C	11·11	N C
3	8·88	N C	8·55	N C
4	10·30	N C	9·96	N C
5	10·77	N C	10·76	Mean of B C & N C
6	9·37	N C	9·43	Mean of B C & N C
7	8·04	N C	7·67	Mean of B C & N C
8	10·02	N C	9·96	Mean of B C & N C
13	3·16	N C	3·01	Mean of B C & N C
5	11·10	B C	10·60	Mean of B C & N C
6	9·21	B C	9·04	Mean of B C & N C
7	8·84	B C	8·53	Mean of B C & N C
8	11·15	B C	10·41	Mean of B C & N C
13	3·11	B C	3·04	Mean of B C & N C
9	10·02	N F	9·72	B C
10	7·11	N F	6·86	B C
11	6·75	N F	6·42	B C
12	8·30	N F	8·24	B C
13	3·04	N F	3·03	Mean of B C & N C

N = Newton's colorimetric reagent.
B = Benedict's colorimetric reagent.
C = Christman & Ravwitch's cyanide carbonate urea solution.
F = Folin's carbonate-free cyanide urea solution.

While under the conditions employed the proportionality was not exact, it was practically the same for the two colorimetric reagents. When Newton's reagent combined with either Christman's or Folin's cyanide solution is used directly on the extracts the method is at least as specific as that of St John & Johnson [1931]. It seems that the rather heroic efforts of Folin and Benedict and their co-workers to prepare a direct uric acid colorimetric reagent, if not altogether successful for blood estimations, have at least produced a technique of practical value for direct determinations on avian excreta. On the other hand, the indirect method is more reliable and should be employed as the standard procedure. Where, however, saving of time is important, and it is known that the direct and indirect methods agree in the estimation of uric acid in excreta obtained from birds on a ration of the same type as that under investigation, the direct method may be of considerable practical value. Close agreement between direct and indirect methods has been obtained also by Shannon [1938], working on fowl's urine which has been separated from the faeces.

Experiments have been carried out to test whether there is any difference between the results obtained in the indirect method when either Benedict's or Newton's reagent is combined with Christman's cyanide solution. The results are given in Table 5.

Table 5

No. of sample	% uric acid on air-dried wt.	
	Benedict's reagent	Newton's reagent
5	10·74	10·63
6	9·22	9·26
7	8·00	8·21
8	10·29	10·08

There was no significant difference between the results obtained by the two methods. In general it was found that Newton's reagent with Christman's cyanide solution did not give such close agreement between duplicates as the Benedict and Franke reagent.

Finally, a number of samples of fowl's excreta have been analysed by the author and other workers in this laboratory by the modification of Woodman's method used here hitherto; the results were compared with those obtained by the standard indirect colorimetric procedure and are given in Table 6.

Table 6

No. of sample	% uric acid on air-dried wt.	
	Colorimetric method	Woodman's method
18	9·61	8·98
19	8·46	9·04
20	9·92	8·66
21	7·35	6·48
22	8·20	7·90
23	8·19	7·17
24	9·78	7·18
25	6·84	6·04
26	6·51	6·03

The results by Woodman's method are in most instances considerably lower than those obtained by the colorimetric method. This is presumably due to the fact that the destruction of uric acid brought about by boiling with lithia more than compensates for any increase in the apparent value caused by nitrogenous impurities which may be present in the uric acid precipitate.

Summary

A colorimetric method is described for the determination of uric acid in fowl's excreta; it is both more specific and less laborious than the methods at present in use.

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12. SEPARATION OF THE HIGHER MONOAMINO-ACIDS BY COUNTER-CURRENT LIQUID-LIQUID EXTRACTION: THE AMINO-ACID COMPOSITION OF WOOL

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Introduction

The possibility of employing counter-current liquid-liquid extraction for the separation of amino-acids in the form of acyl derivatives from protein hydrolysates was briefly discussed by Synge [1939, 1–4], who described preliminary experiments on the separation of some of the naturally occurring 'monoamino-acids'. In the present paper we review the literature on counter-current liquid-liquid extraction, discuss the mathematical and physical basis of the separation of acylamino-acids by liquid-liquid extraction, and describe the construction, operation and testing of a multi-plate chloroform-water counter-current extraction train. We then describe the use of this machine in a control analysis of a known mixture of amino-acids for such components as methionine, valine, proline, the leucines and phenylalanine, together with the application of the new technique to an analysis of wool for these amino-acids. In conclusion we compare the results with those obtained by other methods, and summarize existing knowledge of the amino-acid composition of wool.

Historical

The development of a mathematical treatment of the problems of continuous fractional distillation has made clear the analogy that exists between all counter-current processes. Graphical methods are generally employed, two of the most important being those of Ponchon [1921; cf. Thiele, 1935] and of McCabe & Thiele [1925]. Randall & Longtin [1938, 1–3; 1939, 1–3] have pointed out that the McCabe-Thiele diagram is a point-line contact transformation of the Ponchon diagram, which latter is for many purposes more convenient. These authors have generalized the method of Ponchon for dealing with counter-current processes of all kinds, including liquid-liquid extraction.

Although extraction by immiscible solvents has long been one of the standard purification techniques of the organic chemist, the advantages offered by the application of counter-current principles to this process are only at the present day beginning to be recognized. Nearly all development in this field has so far been in connexion with industrial processes.

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The advantages of this technique, as against distillation, are chiefly these:

(1) Non-volatile and thermolabile materials may be fractionated.

(2) Very small quantities of material may be fractionated by employing dilute solutions. In distillation this is impossible unless a suitable 'carrier' substance is available.

(3) In extractional fractionation partition coefficients—a function of the balance of polar and non-polar groups in the molecule—are the determining property. In distillation, volatility determines the separation. This renders, in many cases, fewer theoretical plates necessary for extractional than for distillatory separation.

Lewis [1916] discussed the application of counter-current principles to the extraction of solids and gases by liquids. Hunter & Nash [1932] applied the McCabe-Thiele diagram to counter-current liquid-liquid extraction. The analogy between this process and distillation was also recognized by Saal & van Dijck [1933], who applied the method of Ponchon to the usual triangular phase diagram for ternary systems. This was also done by Hunter & Nash [1934]. Evans [1934] and Thiele [1935] have discussed the analogy between distillation and extraction, and reviewed previous theoretical treatments of extraction processes. Thiele points out the analogy between extraction columns with successive mixing and settling chambers and plate distilling columns, and shows how continuous-contacting extraction columns (packed or spray types) can be treated by making use of the H.E.T.P. concept, as for packed distilling columns.

During this period counter-current liquid-liquid extraction has become of great importance in the refining of lubricating oils; extraction by liquid sulphur dioxide, nitrobenzene and cresylic acid-liquid propane ('duo-sol' process) have been employed. The efficiency of packed and spray towers has been accurately determined (cf. 'Symposium on Absorption and Extraction', *J. industr. Engng Chem.* **29** (1937), and elsewhere in the same journal).

So far the industrial applications of liquid-liquid extraction have not necessitated columns of very high 'plate number', and in most cases spray or packed columns (which have much greater H.E.T.P. than when used for distillation), or a few mixing and settling units in series have been employed.

The only published work describing a 'multi-plate' column suitable for laboratory use is by Cornish *et al.* [1934], who constructed a counter-current column of 210 units, each with a plate efficiency of about 50 % under the conditions of operation. The apparatus had alternate stirring and settling chambers, and was made of nickel. It was used for the fractionation of fat-soluble vitamins between such solvents as octane and methyl alcohol. The procedure employed was to inject the material to be fractionated at the middle of the column at the beginning of the run, and at the end of the run to collect the two fractions that had flowed out at either end and that which was left in the column.

This procedure differs from all the industrial processes described above, which are essentially continuous. Batch operation necessitates a different theoretical treatment, and Cornish and co-workers obtained an expression for the proportion of material left in the column after a given time of running where the number of theoretical plates, rates of liquid flow and volume of solvents per theoretical plate are given, where the solute is distributed between the two phases according to Henry's distribution law, and where the flow ratio of the two solvents is the inverse of the partition coefficient of the solute under consideration. Under these circumstances the movement of the solute is equally towards each end of the column, and is directly analogous to the flow of heat along a bar.

In connexion with the present work, where a similar mode of operation was adopted, Daniels & Martin, in a forthcoming paper, have generalized and extended the theoretical treatment to deal with solutes obeying Henry's law, but whose partition coefficients differ from the inverse flow ratio of the solvents. They have incidentally shown that the distribution of solute that emerges at opposite ends of the column after an infinite time of running is the same as that which would be obtained under conditions of continuous operation, as calculated from the McCabe-Thiele or similar diagram.

One of us (A. J. P. M.) (unpublished work carried out at the Dunn Nutritional Laboratory, Cambridge) has constructed a glass multi-plate column working with the phases of a petroleum-methyl alcohol mixture, and has applied it to the purification of vitamin E.

Physical chemistry of the separation

In the present work we have separated acetamino-acids by partition between chloroform and water phases. The reasons for using the acetyl derivatives, and some data on their distribution between immiscible solvents, have been given by Synge [1939, 1, 2]. We have used the solvent pair chloroform-water because of the considerable differences between the partition coefficients of acetamino-acids given by these solvents. In addition, the absolute values of these partition coefficients (which determine the flow ratios employed in the column) lie in a suitable range. This is not the case for carbon tetrachloride and water, between which solvents *N*-acetyl-*dl*-leucine has $P = 130$ (37°, $c = 3{\cdot}2$).

Here and subsequently,

c = concentration of solute in aqueous phase, expressed as mg. per ml.,

$$P = \frac{\text{Concentration of solute in aqueous phase}}{\text{Concentration of solute in organic solvent phase}}.$$

An investigation of the distribution of acetamino-acids between petroleum-methyl alcohol phases showed very little 'spread' of the partition coefficients, and these had a very high temperature coefficient. It appeared that the acetamino-acid distribution followed very closely the distribution of methyl alcohol between the phases.

The use of chloroform-water had other secondary advantages: the phases separate readily, having a large difference in density and a high surface tension. In addition, B.P. chloroform, containing a trace of alcohol, does not readily undergo decomposition; we have never detected any liberation of Cl^- into the aqueous phase. Chloroform is non-inflammable. The operation of the column is not affected by atmospheric moisture, as when an anhydrous solvent pair is employed.

In view of the considerable temperature coefficient of the partition coefficients, it was necessary to maintain the column at a constant temperature. The highest practicable working temperature (37°) was employed, as this renders the acetamino-acids more readily extractable into chloroform, and thus allows the column to be operated at convenient flow ratios.

In practice, the acetamino-acids have always been present in dilute solution, and since the relative volumes of chloroform and water phases are known to be scarcely affected by the addition of acetic acid up to a concentration of 5%, we conclude that the same holds good for acetamino-acids, and in practice have found this conclusion justified. This makes possible treatment of the equilibria of each ternary system on a simple two-coordinate diagram, and renders the usual triangular diagram with tie-lines unnecessary. The coordinates

employed have been concentration of solute (mg. per ml.) in the chloroform and water phases respectively.

The equilibria concerned have not been investigated experimentally in great detail, although there has been some addition to and revision of the data given by Synge [1939, 1]. This is appended to the present section.

It is clear that, at a given temperature, two distinct factors will cause the equilibrium curve for a carboxylic acid in chloroform-water to depart from the straight line obtaining under Henry's law. These are (1) ionic dissociation of the solute in the aqueous phase, and (2) association of the solute to double molecules in the chloroform phase.

Smith & White [1929] have investigated the distribution of various carboxylic acids between organic solvents (including chloroform) and water, and have shown that the experimentally obtained curves agree very closely with those calculated by applying the mass action law to the dissociation in the aqueous phase and association in the organic solvent phase, on the assumption that unionized single molecules are distributed between the phases according to Henry's law.

In the present investigation we have studied the distribution of acetyl-*dl*-leucine between chloroform and water phases at 37°.

Assuming an ionization constant of $2{\cdot}3 \times 10^{-4}$ (as recorded for acetylglycine in Landolt-Börnstein's 'Tabellen') it is possible to calculate the concentration of unionized acetylleucine in the aqueous phase from its total concentration, according to Ostwald's dilution law. It will be seen from Table 1 that the ratio

$$D = \frac{\text{Concentration of unionized solute in aqueous phase}}{\text{Concentration of solute in chloroform phase}},$$

remains, within the experimental error, constant over the range studied. No accurate value can therefore be given from these data for the association constant for acetylleucine in chloroform.

Table 1. *Distribution of acetyl-*dl*-leucine between chloroform and water at 37°*

Acetylleucine in water phase millimol./ml.	Acetylleucine in $CHCl_3$ phase millimol./ml.	Percentage ionization	Unionized acetylleucine in water phase millimol./ml.	D
0·0442	0·00462	7·2	0·0410	8·9
0·0242	0·00227	10·0	0·0218	9·6
0·0200	0·00210	10·7	0·0179	8·5
0·0118	0·00104	14·0	0·0102	9·8
0·0055	0·00048	20·5	0·0044	9·2

From this it is clear that in dilute chloroform solution association of acetylleucine to double molecules is insignificant. This is not necessarily the case for other acetamino-acids, although Smith & White have shown (Table 2) that for the series of fatty acids in chloroform the association equilibrium constant is of the same order throughout. In this table

$$K = \frac{(\text{Conc. single molecules})^2}{\text{Conc. double molecules}} \text{ as mg.mol. per ml.}$$

Table 2. *Association of fatty acids in chloroform at 25°*

Acid	K	Acid	K
Propionic	15·3	—	—
Butyric	10·1	*iso*Butyric	9·1
Valeric	10·1	*iso*Valeric	7·6
Hexoic	17·0	*iso*Hexoic	9·2

It is interesting to note that Smith & White found K for α-bromopropionic acid to be 3900, i.e. that association to double molecules is much inhibited by the presence of the α-bromo group. It is possible that the α-acetamino group has the same effect.

We have compared the partition coefficients of optically active and racemic acetamino-acids in one case only (Table 3). Here no difference was observed.

Table 3. *Distribution of acetyl*-d(+)-*leucine between chloroform and water at 37° (cf. Table 1)*

Acetylleucine in water phase millimol./ml.	Acetylleucine in $CHCl_3$ phase millimol./ml.	Percentage ionization	Unionized acetylleucine in water phase millimol./ml.	D
0·0132	0·00125	13·8	0·0114	9·1
0·0069	0·00068	18·5	0·0056	8·3

We considered at one stage the possibility of using acetic acid as a solvent for acetamino-acid mixtures during injection into the chloroform-water extraction train. A preliminary experiment on the effect of acetic acid on the distribution of acetylleucine between chloroform and water phases showed that it caused the acetylleucine to enter the chloroform more readily. The results of this experiment are summarized in Table 4.

Table 4. *Effect of acetic acid on the distribution of acetyl*-dl-*leucine between chloroform and water phases at 37°*

The system contained 10 ml. water, 30 ml. chloroform and 56·2 mg. acetyl-*dl*-leucine, to which was added before equilibration the amount of acetic acid shown in the first column of the table.

Acetic acid added mg.	Acetylleucine in water phase mg./ml.	Acetylleucine in chloroform phase mg./ml.	P
0	4·18	0·393	10·6
50	4·20	0·497	8·45
150	3·98	0·500	7·96
400	3·58	0·700	5·11

This effect is presumably due to the formation of the double molecule ($CH_3.COOH.(CH_3)_2.CH.CH_2.CHNHAc.COOH$) in the chloroform phase.

The presence of considerable amounts of acetic acid in the extraction of acetylated protein hydrolysates and amino-acid mixtures by chloroform in the Neuberger [1938, 1] apparatus [cf. Synge, 1939, 2] is thus seen to be of assistance to the extraction, which is also aided by the large quantities of salt present in the aqueous phase.

In applying these equilibrium data to mixtures of acetamino-acids, the following points must be made:

(1) The degree of ionization in the aqueous phase will depend on the total concentration of acetamino-acid present in that phase. If the ionization constants of all the acetamino-acids present are the same, the degree of ionization of each component of the mixture will be the same, and will depend on the total molar concentration of the components. Thus the ratio of the partition coefficients will be unaffected by dilution, although the absolute value will change.

(2) Association to double molecules in the chloroform phase will, where it occurs, render the substances more readily extractable into chloroform. In mixtures, 'mixed double molecules' may be expected, and the equilibrium

constant for their formation is not predictable. Since the effect seems likely to be small compared with the ionization effect under the conditions used by us, we have not subjected this question to experiment.

In the light of these equilibrium data, it is possible to calculate what degree of separation may be expected when a mixture of two acetamino-acids is fractionated in a multi-plate chloroform-water extraction train. In designing the column described in the present paper we employed the McCabe-Thiele diagram for estimating the number of plates necessary to give the required separation. The following example will illustrate its use.

A column of forty theoretical plates with central feed of an undiluted 1 : 1 mixture of acetyl-*dl*-leucine and acetyl-*l*-proline hydrate is assumed, as this has some bearing on our own experiments. The column is operated at 37°. At this temperature, D for acetyl-*dl*-leucine is 9·2, for acetyl-*l*-proline 17·2 (mean value of determinations). The equilibrium curves for these two substances are plotted separately (Figs. 1 and 2), assuming the ionization in the aqueous phase to follow Ostwald's dilution law, and to have the ionization constant $2{\cdot}3 \times 10^{-4}\ M$.

The flow ratio employed is the geometric mean of the two values of D, i.e. $\frac{\text{Rate of flow of chloroform}}{\text{Rate of flow of water}} = 12{\cdot}58$, and is taken as constant throughout the column.

McCabe-Thiele diagrams are constructed for each solute separately (Figs. 1 and 2). The location of the operating lines is determined as follows:

(1) Both lines (rectifying and analysing) are parallel, and have a slope of 1/12·58.

(2) The operating lines cut the axes at the concentration of solute in the chloroform and water outflows respectively.

(3) Twenty theoretical plates may be drawn between each operating line and the equilibrium line from the point at which the operating line cuts the axis to the point on the equilibrium line corresponding to the feed-plate.

In Figs. 1 and 2 the separation of the two solutes is treated individually. In Fig. 3 the individual and total concentrations of the two solutes on each plate, as given by Figs. 1 and 2, are plotted.

It will be seen that the curvature of the equilibrium lines in very dilute solution causes the acetylproline to flow out almost completely in the water outflow, whereas only 97·6 % of the acetylleucine flows out in the chloroform, and the rest in the water.

Since, however, the degree of ionization will depend on the total concentration of acid in each plate, it will be seen from Fig. 3 that this does not fall below 0·02 M, and the degree of ionization will therefore at no point of the column be greater than 10 %. The equilibrium lines will therefore approximate more closely to straight lines through the origin, and the separation will be more nearly that calculated on the assumption that Henry's law operates, i.e. 99·6 % of each solute will emerge from the column at one end and 0·4 % at the other.

Under conditions of batch operation, where the mixture to be fractionated is added in one lot initially at the middle of the column, the separation cannot be so readily predicted, although the work of Daniels & Martin suggests that it should not differ widely from that obtained under conditions of continuous operation.

In batch operation, dilution will cause any component of the mixture to move more readily towards the water outflow end of the column; complete removal will therefore be more readily obtainable for the components of the mixture flowing out largely in the water than for those flowing out largely in the chloroform. We therefore intend in future to operate the column in a manner

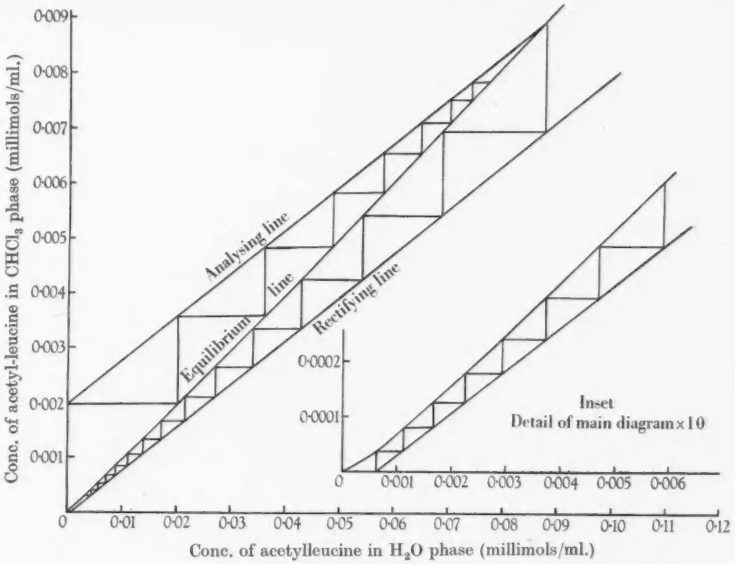


Fig. 1. McCable-Thiele diagram showing distribution of acetylleucine.

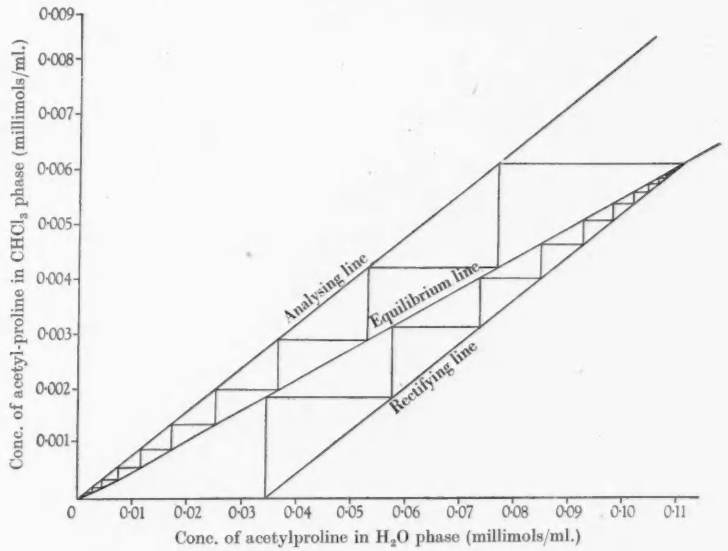


Fig. 2. McCabe-Thiele diagram showing distribution of acetylproline.

directly analogous to batch fractional distillation, i.e. by adding the mixture initially at the chloroform outflow end of the column, and maintaining its concentration there by evaporation of solvent without withdrawal of solution. Gradual increase of the rate of water flow through the column should then lead to sharp fractionation of the material obtained in the water outflow from the

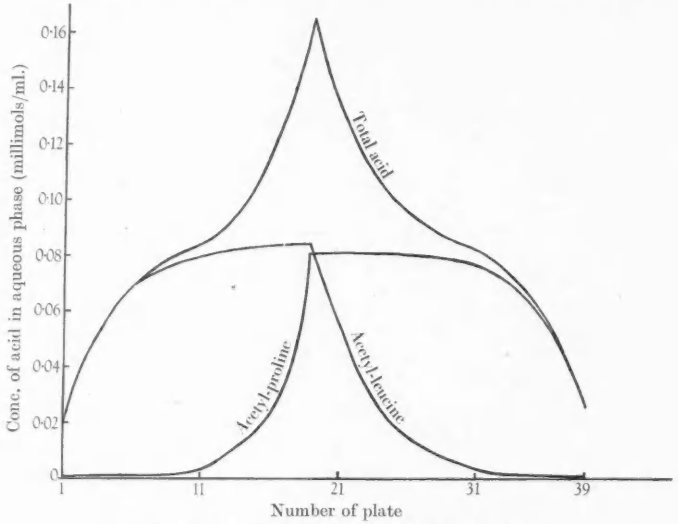


Fig. 3. Distribution of acetamino-acids in the column.

column. In addition, the effective length of the column would be twice that under the conditions of operation described in the present paper, since the column would be completely rectifying, whereas under the present conditions each half of the column serves as analyser for the rectifying action of the other half.

Table 5. *Partition of acetamino-acids between chloroform and water phases at 37°*

Compound	Conc. in water phase mg./ml.	Conc. in chloroform phase mg./ml.	P
Acetyl-*l*-phenylalanine	1·44	0·37	3·9
	0·76	0·18	4·2
Acetyl-*d*-*nor*leucine	1·74	0·27	6·45
	0·94	0·13	7·2
Acetyl-*d*-*iso*leucine	1·86	0·25	7·4
	0·98	0·11	9·0
Acetyl-*d*-leucine	2·28	0·22	10·5
	1·20	0·12	10·0
Acetyl-*l*-valine	3·3	0·22	15
Acetyl-*l*-proline (hydrate)	3·03	0·16	18·9
	1·55	0·09	17·2
Acetyl-*l*-methionine	3·04	0·11	27·6
	1·46	0·045	32·4
Acetyl-*dl*-α-aminobutyric acid	3·42	0·05	68
Acetyl-*dl*-alanine	4·74	0·022	216

New data on the partition of acetamino-acids between chloroform and water

In connexion with the present work it was desirable to redetermine and supplement some of the data for partition coefficients given by Synge [1939, 1]. The figures given in Table 5 were obtained by gravimetric determination of the solute in each phase after equilibration in a separating funnel.

The main discrepancy between the present figures and those previously given is in the figure for valine. The actual separations described below gave valine a place intermediate between proline and methionine. It is possible that the partition coefficient of its acetyl derivative alters more rapidly on dilution than that of proline.

Description of the machine

In the apparatus employed for the present work, the column in which water and chloroform phases flow counter-current consists of 40 units in series. Fig. 4 illustrates a single unit. The vertical or 'stirring' tubes (1, Figs. 4 *B*, 5, 6, 7) and

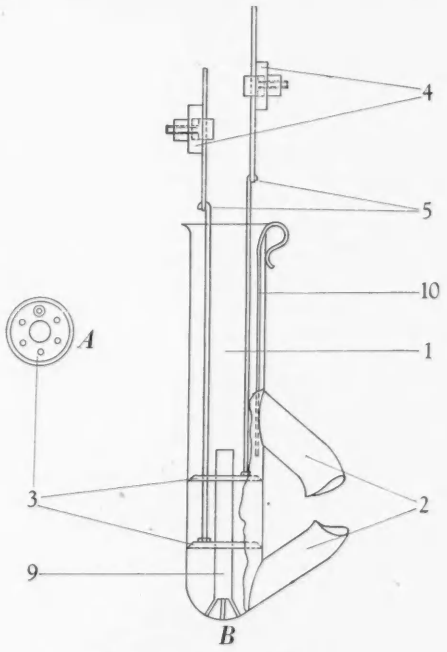


Fig. 4. Single unit of column.

the inclined or 'settling' tubes (2, Figs. 4 *B*, 5, 6, 7) connecting successive stirring tubes are made of pyrex glass. Each stirring tube holds a pair of silver stirrers (3, Fig. 4), each being a perforated dished disk of 0·017 in. silver sheet riveted to hard-drawn 16 s.w.g. silver wire. (Silver, unlike nickel and silver solder, is not attacked under the conditions of operation of the column.)

Each stirrer is attached to a grid (4, Figs. 4 *B*, 5, 6, 7) through a universal joint (5, Figs. 4 *B*, 7). The two grids, and thus each pair of stirrers, are given equal and opposite reciprocating motions by a system of links (Watt's parallel motion) (6, Figs. 5, 6, 7). This is driven through a variable-throw crank (7, Fig. 8) and sprocket gear by a 1/4 H.P. induction motor (8, Fig. 8) mounted below the table.

Fig. 5.

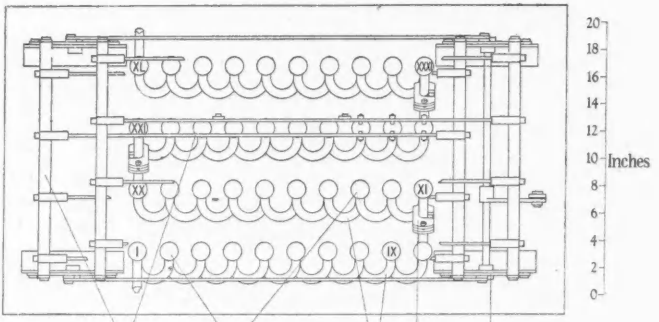


Fig. 6.

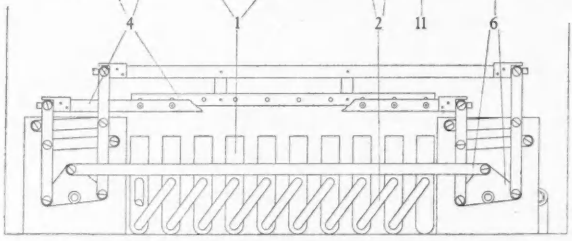


Fig. 5. Plan of column. Fig. 6. Side elevation of column.

A vertical glass tube (9, Fig. 4 *B*), open top and bottom and having three feet, rests on the bottom of each stirring tube and passes through the central perforation of each stirrer. A baffle plate (10, Fig. 4 *B*) of silver sheet is clipped to the rim of each stirring tube, and covers the mouth of the settling tube.

The stirring tubes are numbered 1–40 as shown in Fig. 5, and are sealed together in four banks of ten elements each. Successive banks are connected through a ground-glass flange joint, having a cellophane washer and held in position by an ebonite clamp (11, Figs. 5, 7). The chloroform and water entries (12 and 13) and exits (14 and 15) are arranged as shown in Figs. 7 and 8.

The column rests on a stoutly built table, and is held in position by light clips (not shown in drawing). The link-gear and the whole column (except for the water and chloroform exits) is enclosed in a wooden casing (16, Figs. 7, 8) resting on the table. The interior of this is maintained at 37° by thermostatically controlled electric lamps. The air is kept in circulation by an electric fan. The casing is provided with a lid (17, Fig. 8), windows (18, Fig. 8) and a small door near to tube 20.

On leaving the column, chloroform flows from the adjustable level outflow (14, Figs. 7, 8) through a tube provided with a tap for sampling (19, Fig. 8) to the chloroform recovery still, a 2 l. flask (20, Fig. 8) which is immersed in an

electrically heated water bath (1 kW.). The vapour from the still passes through the cyclone trap (21, Fig. 8), which eliminates entrainment of drops of liquid and, after condensation in the reflux condenser (22, Fig. 8), the chloroform falls to the constant level vessel (23, Fig. 8). The reflux arrangement is adopted so that the chloroform shall enter the constant level vessel hot, and thus free from dissolved air. This prevents the formation of bubbles in the flowmeter.

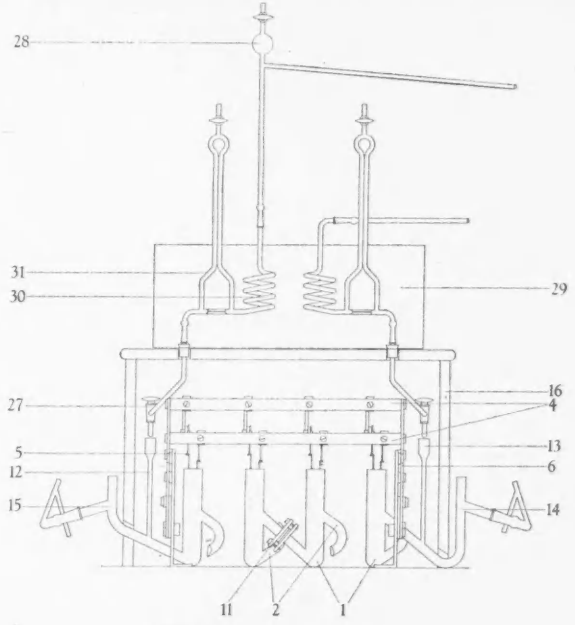


Fig. 7. End elevation of column.

The level of liquid in the constant level vessel is adjusted by raising or lowering the air inlet tube of the 10 l. Mariotte bottle (24, Fig. 8) which acts as the chloroform reservoir. The constant level vessel is connected to the reservoir and to the flowmeter through siphon tubes (25, Fig. 8); the siphons are maintained by differential pressure provided by a column of chloroform in the wash-bottle (26, Fig. 8) through which air is withdrawn from the Mariotte bottle. Chloroform is admitted to the column through the tap (27, Fig. 7). The rate of admission of chloroform is controlled by adjustment of the tap and of the chloroform level in the constant level vessel. Any water separating from the chloroform on its way to the column as a result of cooling is removed in the trap (28, Figs. 7, 8) below which the chloroform supply tube enters the water bath (29, Figs. 7, 8), which is thermostatically maintained at 37°. The chloroform passes through the heating coil (30, Fig. 7) and the capillary flowmeter (31, Figs. 7, 8) before passing to the column.

The same arrangement (not shown in drawing) is used for supplying water to the column; here de-aeration is effected by an electric immersion heater (60 W.) placed in the constant level vessel.

The gear for driving the stirrers was constructed for us by Unicam Instruments, Ltd., Cambridge, and the glass part of the column was made by Messrs A. Gallenkamp, London. We are grateful to their glass-blower, Mr J. Holme, for the efficient execution of this work. We wish also to thank Messrs H. Hall and E. Benton, of the Biochemical Laboratory, Cambridge, and Mr S. Dilworth of Torridon for their help in building the apparatus.

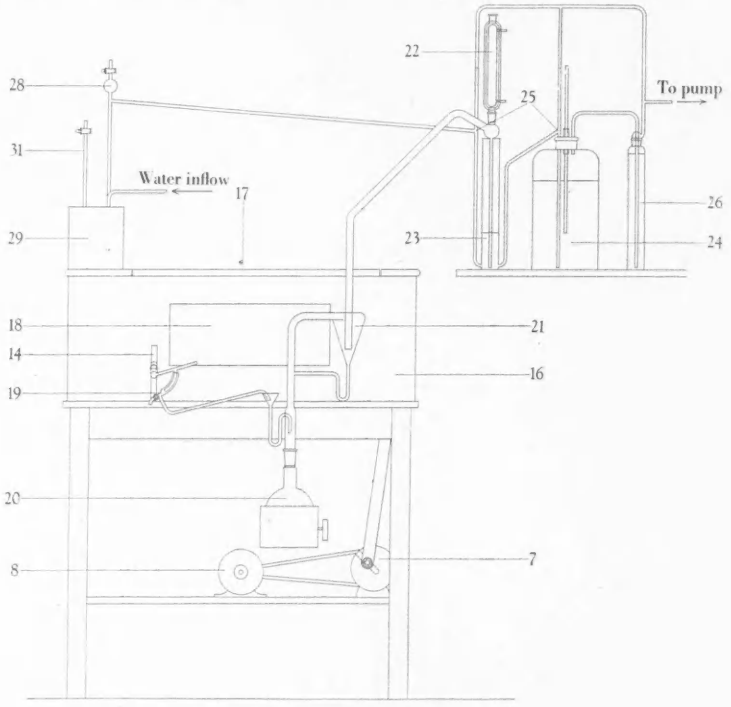


Fig. 8. General view of apparatus.

Operation of the column

When the column is in action, the motion of the stirrers maintains in each stirring tube an emulsion of which the aqueous is the disperse phase. Drops of water thus rise through the settling tube which leads to the next stirring tube. In this way a continuous stream of water passes along the column from tube 1 to tube 40. At the same time, chloroform flows in the opposite direction; under constant conditions the flow of each solvent will be uniform along the whole length of the train, and will depend on the rate at which the solvents are admitted to the column. The solvents leave the column by the adjustable-level outflows.

It will be appreciated that in addition to their normal function of contacting the liquids, the stirrers serve to pump the liquids along the column by continually displacing, in the stirring tubes, the chloroform and water phases from the

positions that they would occupy at rest, thus providing gravitational potential energy for the settling process. This feature of design makes it unnecessary for the column to be tilted [cf. Cornish *et al.* 1934] and thus makes possible the present compact arrangement.

Although the stirrers in each tube are always moving equally in opposite directions, they tend to have, in addition to their stirring action, a 'dynamic' pumping action, which depends on:

(1) The orientation of the dished rim of the stirrers, as there is greater resistance to the motion of the stirrer in one direction than in the other.

(2) The asymmetry of motion of the link gear: the motion is not simple harmonic, and the stirrers approach each other at a different speed from that at which they separate. This effect can be reversed by attaching each stirrer to the other bar.

(3) The length of stroke and velocity of the stirrers, being greater for long strokes and high velocities.

(4) Possible differences in the viscosity of the emulsion at the different levels in the tube at which each stirrer is situated.

This pumping effect, being additive from tube to tube, can, if present in the same sense in a succession of tubes, so alter the level of liquid at different points in the column as to render it inoperative. It is greatly reduced by the central glass tubes, which prevent the establishment of serious pressure differences across any pair of stirrers, and are included for this reason as well as for convenience in emptying the column.

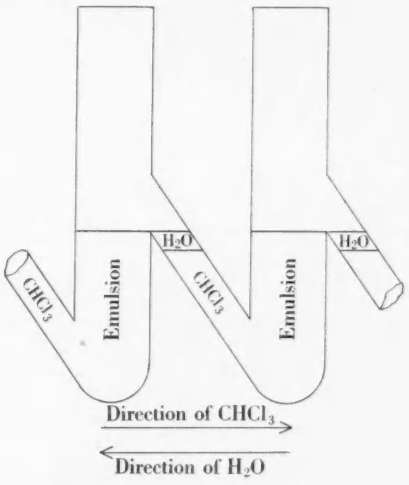


Fig. 9. Normal running of column.

If, as is the case when water is the disperse phase, the density of the emulsion in a stirring tube is less than that of the emulsion in the settling tube, there will be a tendency to 'static' pumping of liquid along the column towards tube 1. The level of the top of the emulsion in each stirring tube will thus tend to its low limit, i.e. the level of the lip of the settling tube leading out of it, as shown in Fig. 9. In practice, at low flow rates of chloroform, the surface can fall lower

than this by splashing of chloroform over the lip into the settling tube. In this way the surface level of the liquid may fall to the level of the top stirrer, and splashing then becomes so bad that liquid is actually lost from the column. This is prevented by placing a silver baffle plate in front of the mouth of each settling tube, which checks the passage of liquid by splashing, and at the same time serves to stop 'entrainment' of chloroform by back-mixing between successive units. Thus, at low flow rates of chloroform, each settling tube will have a layer of water at its top and each stirring tube will be full to the lip of the settling tube (Fig. 9), so that the average density of liquid in each limb is equal.

Under these conditions, the level of liquid in each stirring tube will be at its low limit, and this is the most desirable condition in which to operate the column. Stirring is less efficient when the level of the liquid in the stirring tubes rises, as is bound to happen on increasing the rate of chloroform flow, owing to the head required for overcoming the resistance of the column to flow. This rate can, however, be increased without impairing the operation of the column until it cancels out the effect of the 'static pumping', i.e. until there is no separated layer of water at the top of the settling tubes.

The rate of passage of water out of each stirring tube, and thus along the column, is normally determined by the bottom aperture of the corresponding settling tube, by the opposed rate of flow of chloroform (increase of which decreases the speed of settling), and by the concentration and state of division of the water phase at the bottom of the stirring tube. These depend on the efficiency of the stirring and on the surface tension of the emulsion, which may be lowered by the presence of dissolved substances. If, as occasionally happens, the presence of these causes excessive emulsification, the rate of flow of solvents may be determined entirely by the rate of settling in the settling tubes.

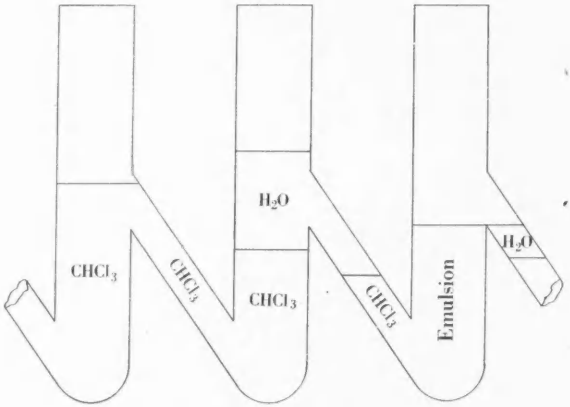


Fig. 10. Water blockage of column.

In order to get efficient stirring throughout the tube, the vertical position of the stirrers must be carefully adjusted. This is particularly necessary when the stirrers are operating on a short stroke (at correspondingly greater speed); these are the most favourable conditions for the elimination of 'dynamic' pumping and splashing.

When more water enters a stirring tube than it is capable of passing, water collects at the top of the tube, raising the level in the tube and preventing the flow of chloroform. This causes accumulation of chloroform between the defective unit and the chloroform inflow, as shown in Fig. 10. This type of blockage is more difficult to deal with than a block due to excessive flow of chloroform, as the column responds more slowly to changes in the rate of water inflow than to changes in the rate of chloroform inflow. Moreover, in the course of eliminating a 'water block' (and sometimes in ordinary operation, when water-flow is being established through the column) the column may exhibit a cyclical instability, since the removal of the 'water block' may be followed by a period of intense 'static pumping', followed by the re-establishment of the block farther along the column. These phenomena involve fluctuations in the flow ratio at different points of the column, and are avoided as far as possible by working with a low level of liquid in the column and moderate rates of solvent-flow.

Working and testing the machine

In working the machine, we proceed as follows. The water and air thermostats being at 37°, chloroform is admitted until the column is full. The rate of admission of chloroform to the column is then adjusted, and the stirrers are set in motion (stroke 0·75 cm. at 390 vibrations per min.). The required flow of water is then started. After running in this manner for some hours, constant conditions will be established throughout the column. Since, however, the addition of the material to be analysed lowers the surface tension between the phases, thereby increasing emulsification and the rate of flow of water, the material is usually added to the column at tube 20 as soon as the advancing stream of water has reached tube 27–29. This immediately liberates water from the middle part of the column, which rapidly finds its way towards tube 40, and an approximately steady state is thus established before water begins to leave the column.

At the end of the run, the material that has emerged in the chloroform is collected from the flask of the chloroform recovery still, while that which has emerged in the water is obtained by concentration *in vacuo*. The fraction remaining in the column is obtained by emptying the column and concentrating separately the two phases so obtained.

Although Cherbuliez & Wahl [1925] have found benzamino-acids to be volatile in steam, we have examined for N the distillates obtained in the concentration of solutions of acetamino-acids with negative results.

The solvent volumes in the column are nearly independent of the flow ratio employed. 3·0–3·5 l. of chloroform and 150–350 ml. of water are the usual amounts.

Satisfactory rates of solvent flow for the range of flow ratios employed are shown in Table 6.

Table 6. *Solvent rates for different flow ratios in the column*

Flow ratio	Chloroform rate ml./min.	Water rate ml./min.
68	22·5	0·33
20·8	18·75	0·86
13·9	19	1·37
8·0	8·0	1·00

Since on the average each molecule of chloroform or water will spend about 3 min. in each unit of the column, and as intimacy of mixing in the stirring tubes is greater than in the ordinary laboratory separating funnel, it seems likely that each unit will correspond nearly to one theoretical plate, assuming no entrainment to occur. By the use of red ink, the pigment of which stays completely in the water phase, we have never detected any entrainment of water, while the use of Sudan III, which stays completely in the chloroform, has shown that entrainment of chloroform is only detectable at the lowest rate of chloroform flow employed; under these circumstances it is not great, and only occurs in the absence of the baffle. The main factor vitiating the performance of the machine would seem to be local variations in the flow ratio, particularly those occurring at the beginning of the run.

Although precautions were taken against loss of chloroform by evaporation by sealing cracks, etc. in the walls of the air-thermostat box with plasticine, by packing wool around the entry of the driving-arm to the box, and by providing a rubber seating for the box lid, they were not completely successful, the total loss of chloroform on a 24 hr. run amounting to about 1·5 l.; this is not surprising when it is remembered that the equilibrium weight of chloroform vapour in the box at 37° is about 500 g. This loss corresponds to about 1 ml. per min. of chloroform, and most of it must be assumed to take place along the length of the column. Its effect on the operation of the column is greatest at low flow ratios, and is to produce a continuous change of flow ratio along the length of the column, amounting, at flow ratio 8, to a change of 12·5%.

In operating the machine in most of this work we placed the material to be analysed in the middle of the column in solid form in one batch. Larger quantities could be added in a continuous manner; the machine is just as capable of continuous as of batch operation. For this purpose tube 20 is connected through a capillary to an electrically driven glass syringe. The problem is to find a suitable solvent for the acetamino-acids which will permit the slow injection of a concentrated solution. The mixtures obtained from proteins crystallize from concentrated aqueous solution, and glacial acetic acid, though a good solvent, distils with the chloroform, and thus accumulates in the column, seriously affecting the equilibria, as already pointed out. This effect is strikingly demonstrated by comparing the results of tests 1 and 2 given below.

Test 1 is also of interest because the time course of emergence of the substances to be separated was studied. In other experiments we have only collected the three fractions at the end of the run.

Possibly alcohol would prove a more desirable solvent for injection.

Tests. For testing the machine, a 1 : 1 mixture of acetyl-*l*-proline hydrate [du Vigneaud & Meyer, 1932, 1, 2] and acetyl-*dl*-leucine [Fischer, 1901] was employed. These substances have the partition coefficients given in Tables 1 and 5, which are sufficiently close to give a good test of the machine, and in addition small amounts of each in the presence of the other can be conveniently detected, the former by its large negative rotation, and the latter by determination of amino-N after acid hydrolysis.

Test 1. The column was allowed to reach a steady state at flow ratio 13·9 (solvent rates as in Table 6). A solution of 4·5 g. of acetyl-*l*-proline hydrate and 4·5 g. of acetyl-*dl*-leucine in 25 g. of glacial acetic acid was then injected at a steady rate, 12 hr. being required for complete injection. The chloroform and water effluents were collected at the intervals shown in Table 7, which shows cumulatively the amount of each solute (as % of amount finally injected) which had emerged at different times.

Table 7

Time from commencement of injection hr.	In chloroform effluent		In water effluent	
	% of acetyl-leucine	% of acetyl-proline	% of acetyl-leucine	% of acetyl-proline
4	0·46	0·0	0·00	1·0
6	2·7	0·1	0·02	1·8
8	14·0	0·6	0·07	3·2
10	40·4	3·4	0·13	5·1
12	65·9	9·3	0·24	6·7
15	92·4	25·2	0·38	8·7
19	98·8	41·2	0·50	11·5

Test 2. Column as in test 1. A mixture of 1·04 g. acetyl-*dl*-leucine and 1·02 g. acetyl-*l*-proline hydrate was added in the solid form, and the machine was allowed to run for 11 hr. At the end of this time there had emerged in the chloroform 26·5 % of the acetylleucine and 0·60 % of the acetylproline. In the same time there had emerged in the water 4·36 % of the acetylleucine and 48·7 % of the acetylproline.

The wide divergence between tests 1 and 2 may be ascribed to the presence of acetic acid in test 1. The accumulation of this has the effect of rendering both solutes more readily extractable by chloroform.

These tests gave a rough idea of what degree of separation was to be expected from the machine, and we were able on this basis to use it for the separation of more complex mixtures.

Separation of a known mixture of amino-acids

The possibility of using liquid-liquid extraction for separating and estimating some of the monoamino-acids occurring in protein hydrolysates was briefly discussed by Synge [1939, 2], who described a procedure for obtaining a good yield of the required amino-acids in the form of their acetyl derivatives and free from salt. Such a mixture is suitable for fractionation in the machine which we describe in this paper.

Before attempting to use the new technique for the analysis of a protein it was considered advisable to control it by experiment with a known mixture of amino-acids.

The preliminary fractionation of such a mixture was described by Synge [1939, 2], who stated: 'The mixture was intended to simulate a gelatin hydrolysate, and lacked cystine and tryptophan. It would obviously be desirable to repeat the experiment with a mixture containing these amino-acids, as they are responsible for much secondary change when such a mixture is boiled in acid solution.'

The present investigation has been conducted with special reference to wool, in which both these amino-acids are present, cystine being a major constituent. A mixture was made up containing all the amino-acids recorded in the literature as constituents of wool, with the exception of methionine and phenylalanine. The former was omitted because its behaviour had already been studied [Synge, 1939, 2] and its absence permitted a study of the behaviour of cystine during the fractionation by means of total S determinations.

The amino-acids were in roughly the proportions recorded for wool.

All samples used for analysis were aliquots; allowance has been made for the samples removed at each stage for analysis, and all figures given are on the basis of the original mixture, as detailed in Table 8.

All evaporations were carried out *in vacuo* below 40°.

The values given for 'amino-N' refer to amino-N determined by the Van Slyke procedure on samples hydrolysed with 2 *N* HCl for 3 hr. at 100°, and subsequently neutralized with excess of sodium acetate. Where the material had been dissolved in alcohol, great care was taken to remove this completely before determining amino-N.

Table 8. *The mixture of amino-acids used in the experiment*

Compound	N (g.)	N as % of total N
Glycine	0·290	12·8
dl-Alanine	0·137	6·0
dl-Valine	0·091	4·0
l-Leucine	0·206	9·0
dl-Serine	0·070	3·1
l-Proline	0·128	5·6
l-Aspartic acid	0·053	2·3
l-Glutamic acid	0·190	8·3
l-Histidine (as hydrochloride)	0·048	2·1
l-Arginine (as hydrochloride)	0·482	21·2
dl-Lysine (as dihydrochloride)	0·109	4·8
l-Tyrosine	0·083	3·6
l-Cystine	0·242	10·7
l-Tryptophan	0·026	1·1
Ammonium chloride	0·124	5·4
Total	2·279	100

The mixture shown in Table 8 was dissolved in 200 ml. 6 *N* HCl, and was refluxed for 24 hr. The resulting solution was concentrated and acetylated exactly as described by Synge [1939, 2], using 400 ml. 2 *N* NaOH and 40 ml. of acetic anhydride. After keeping overnight, the mixture was acidified as described, transferred to the Neuberger extractor, and extracted with approximately 30 vol. of chloroform. The aqueous residue was freed from salt and re-acetylated exactly as described, acidified, and again extracted with 30 vol. of chloroform in the Neuberger extractor. The two chloroform extracts were combined and evaporated to dryness: the residue was made up to standard volume in water (fraction E).

This fraction contained 24·7 % of the total N of the mixture.

It was completely free from $SO_4^=$, and contained 42 mg. S (7·6 % of the cystine-S of the mixture). We are grateful to Mr W. R. Cuthbertson for this S determination.

Fraction E was evaporated to dryness in a vacuum desiccator, and the resulting toffee-like solid was fractionated in the machine for 24 hr. at flow-ratio 68, the geometric mean of the partition coefficients of acetylalanine and acetylproline. In this and subsequent fractionations the solvent rates employed were those given in Table 6.

Table 9 shows the distribution of N, S and amino-N in the three resulting fractions.

Table 9

Fraction	N as % of total N	S as % of original cystine S	Amino-N as % of total N
68_c (in chloroform)	17·9	2·2	12·7
68_m (in column)	0·8	—	—
68_w (in water)	5·9	5·0	—

Fraction 68_w was evaporated to dryness. It was obviously a complicated mixture, and crystallized very slowly. By successive crystallization from ethyl

acetate and acetone a small amount of acetylglycine was isolated. The fraction might be expected to contain also acetylalanine, *N*-acetyltyrosine, diacetylcystine, diacetyllysine and possibly other constituents. It was not further investigated.

Fraction 68$_m$ was not further investigated.

Fraction 68$_c$ was evaporated to dryness, to give a practically completely crystalline residue, coloured pale yellow. It was fractionated in the machine for 24 hr. at flow-ratio 13, giving the fractions shown in Table 10.

Table 10

Fraction	N as % of total N	Amino-N as % of total N
68_c13_c	2·7	—
68_c13_m	3·2	—
68_c13_w	10·2	5·8

68_c13_m and 68_c13_c both obviously consisted almost completely of acetylleucine, and were therefore combined before working up.

The material consisted of crystals accompanied by a brown oil, which was later found always to be produced in the course of fractionation in the machine. Its nature is discussed in the succeeding section dealing with the analysis of wool. It could readily be separated from the crystals by washing them with chloroform or ligroin. By subsequent recrystallization from chloroform and from water, three crops were obtained:

Crop I. 1·25 g. M.P. 182°. $[\alpha]_D^{24°} -23·5°$ (alcohol, $c=3·3$). (Found: C, 56·2; H, 8·54; N, 8·23%. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%.)

Crop II. 0·070 g. M.P. 155–160°. $[\alpha]_D^{24°} -5·2°$ (alcohol, $c=1·0$). (Found: C, 55·9; H, 8·53; N, 7·8%.)

Crop III. 0·162 g. Not further investigated.

Synge [1939, 1] records acetyl-*d*-leucine as having M.P. 186–188°; $[\alpha]_D^{22°} +23·2°$ (alcohol). Fischer [1901] records acetyl-*dl*-leucine as having M.P. 161°.

From this it seems that the crystalline part of this fraction consists of acetyl-*l*-leucine which has undergone about 15% racemization.

89% of the N of fractions 68_c13_m and 68_c13_c is thus accounted for as crystalline acetylleucine.

68_c13_w. On concentrating an aqueous solution of this fraction, the first crop of crystals (0·353 g.) had M.P. 180° and this was not depressed on admixture of acetyl-*l*-leucine from the other two fractions.

The next crop obtained melted at 130–137°, and the next at 110–140°. From these it proved possible, by repeated crystallization from water, to isolate 0·269 g. of a product having M.P. 147–148°. This was not depressed by admixture with authentic acetyl-*dl*-valine [Synge, 1939, 1]. (Found: C, 52·5; H, 8·22; N, 8·90%. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8%.)

Since it was clear that the mother liquors contained a mixture of acetylleucine, acetylvaline and acetylproline, and since acetylproline had already been satisfactorily isolated in the analysis of wool (see below), this fraction was not further investigated. Its proline content could be judged by the difference between total and amino-N, shown in Table 10.

We draw the following conclusions from this experiment with a known mixture of amino-acids:

(1) Fraction E, on fractionation in the machine at flow-ratio 68, yields in fraction 68_c the amino-acids leucine, proline and valine in about 95% yield. It

will be noted that the ratio of amino-N to total N for fraction 68_c is 0·71. Calculated from the original mixture, $\frac{\text{Leucine-N} + \text{valine-N}}{\text{Leucine-N} + \text{valine-N} + \text{proline-N}} = 0{\cdot}704$. Methionine, leucine isomers and phenylalanine may be expected to behave similarly.

(2) Racemization to the extent of about 15% is to be expected. This probably occurs during acetylation, although the conditions of acetylation are so chosen as to reduce it to a minimum [cf. du Vigneaud & Meyer, 1932, 1]. The possibility of racemization of the free amino-acids on prolonged boiling with acid cannot be ignored. With cystine, this is known to be considerable, and Arnow & Opsahl [1940] and Johnson [1940] report slight racemization of glutamic acid under these conditions.

(3) 2–3% of the S of the cystine in the mixture is carried into fraction 68_c. This cannot be in the form of diacetylcystine [cf. Synge, 1939, 1]. The state of combination of this S is discussed in the following section on the analysis of wool under fraction 68_m.

(4) Further fractionation of 68_c at flow ratio 13 largely freed about 70% of the leucine from valine and proline, the bulk of which remained in fraction 68_c13_w. It might be expected that repeated fractionation in the machine at different flow ratios would achieve better separation. This was attempted with some success in the course of the work on the monoamino-acid fraction of wool described below.

Investigation of the higher monoamino-acids of wool

The literature on these constituents of wool is meagre. Abderhalden & Voitinovici [1907] investigated a wool hydrolysate by the ester distillation procedure of Fischer and reported figures for valine, proline and 'leucine' which are of interest in connexion with the present investigation. No attempt was made to decide whether *iso*leucine was present with the leucine. Phenylalanine could not be detected in the fraction in which it was expected to occur. Mueller [1923] described the isolation of methionine in small quantity from wool, and Barritt [1934] employed the volatile iodide method of Baernstein [1932] for its determination. Block [1939] reported a colorimetric determination of phenylalanine in wool. These analyses are compared at the end of this section with the results of the present investigation.

Commercial Merino 64*s* dry-combed top was extracted in a Soxhlet apparatus with benzene and alcohol successively. It was then washed several times with distilled water.

An amount containing 3·310 g. of N (approx. 20 g. of wool) was refluxed on an air bath with 200 ml. of 6*N* HCl for 25 hr. [cf. Marston, 1928].

In describing the subsequent analysis, allowance has been made for the aliquots removed at each stage. Wherever possible, amounts are expressed in N as % of total N of the original wool. Where absolute quantities are mentioned, they are to be referred to the original wool employed, containing 3·310 g. of N. In the later stages of the analysis the amounts of material actually employed corresponded to about 10 g. of wool.

Total N determinations on the various fractions were carried out by the Kjeldahl technique. N determinations on solid material were by the Dumas technique, and were carried out by Dr G. Weiler, Oxford, who is also responsible for the C, H and S determinations.

In the course of the acid hydrolysis a small amount of partly crystalline water-insoluble material was observed to collect in the condenser. It could be

recrystallized from ether, in which it was very soluble. Suspecting the presence of a volatile organic acid, we diluted the hydrolysate when cool to 500 ml. and extracted it with 5 vol. of chloroform in the Neuberger extractor. The material which had collected in the condenser was combined with the hydrolysate before extraction.

An aliquot of the chloroform extract (which was free from Cl^-) was shaken with water, and the mixture was titrated to phenol red with NaOH. The alkali required was equivalent to 0·204 milliequiv. of acid in the whole extract.

Another aliquot of the extract was evaporated at 37° in the open in a tared dish. The material not volatile under these conditions corresponded to 83 mg. in the whole extract.

The rest of the extract was evaporated at atmospheric pressure; the non-volatile residue from this was suspended in water and subjected to steam distillation until 500 ml. of distillate had been collected. The acid not distillable under these conditions amounted to 0·08 milliequiv.

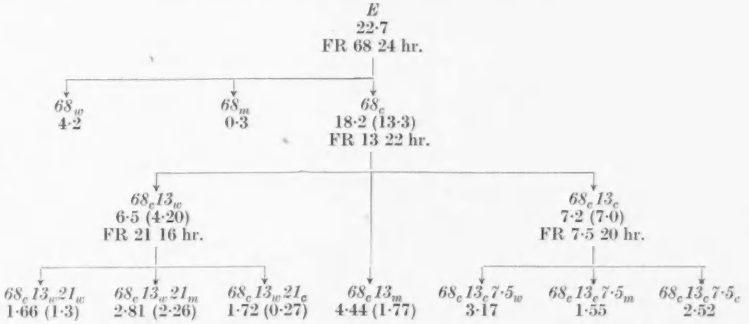
Steam distillation of the extract after neutralization to *p*H 7 with NaOH gave a small amount of water-insoluble material in the distillate.

In view of the insignificant quantities concerned, these fractions were not further investigated. We have indications that the amount of material extractable by chloroform from an acid hydrolysate of wool is increased by increasing the time and temperature of hydrolysis. Most of the material in question is therefore probably a product of secondary change.

After this extraction by chloroform, the hydrolysate was concentrated to dryness *in vacuo*. Two successive acetylations and extractions were carried out exactly as described above for the known mixture, and the combined extracts constituted fraction E, containing 22·7 % of the total N and 54·6 mg. of S (about 8 % of the total S of the wool).

Fraction E was then fractionated and sub-fractionated in the machine according to the scheme shown in Table 11. Between fractionations it was

Table 11. *Sub-fractionation of fraction E*



found convenient to handle the fractions by solution in alcohol. The nomenclature of the fractions is that employed in the previous section. The figures given immediately after each fraction in the table show its N content as % of total N, and the figures in brackets its amino-N content on the same basis, determined as in the previous section.

The subsequent fractionation to which a fraction was subjected is shown in the table thus: *FR 68 24 hr.*

This means that the material in question was fractionated in the machine at flow ratio 68 (with solvent rates as given in Table 6) for 24 hr., after which the three resulting fractions were collected.

Non-amino-N of the fractions. This, the difference between total and amino-N, sets an upper limit to the proline present. It is calculated for the relevant fractions as shown in Table 12. In view of the good yields of crystalline acetyl-proline obtained in the investigation of the end-fractions, there is no reason for postulating the presence of any non-amino-N-containing substance other than proline.

Table 12. *Non-amino-N of the fractions*

Fraction	Non-amino-N as % of total N of wool
$68_c 13_w 21_w$	0·36
$68_c 13_w 21_m$	0·55
$68_c 13_w 21_c$	1·45
$68_c 13_m$	2·67
$68_c 13_c$	0·20
Total	5·23
Cf. 68_c	4·9

Methionine determinations on the fractions. These were kindly carried out for us by Mr G. Lindley, by the 'volatile iodide' method of Baernstein [1932; 1936]. The results are given in Table 13.

Table 13. *Methionine of the fractions*

Fraction	Methionine-N as % of total N of wool
68_c	0·52
$68_c 13_w 21_w$	0·31
$68_c 13_w 21_m$	0·09
$68_c 13_w 21_c$	0·01

Great care was taken to remove alcohol completely from the samples before subjecting them to analysis, since this would appear as volatile iodide in the course of the determination. There remained the possibility that handling the fractions with alcohol had induced partial esterification.

The distribution of volatile iodide in the end-fractions, however, makes it clear that acetamino-acid ethyl esters are not involved, since these are readily extractable from water by chloroform. It is however possible that 25% of the volatile iodide determined on fraction 68_c may be due to this cause.

The final distribution of volatile iodide in the end-fractions is roughly what would be expected from a knowledge of the partition coefficient of acetyl-methionine (cf. Table 5).

Detailed investigation of the end-fractions.

68_w. This did not readily crystallize, and being obviously a complex mixture was not further investigated.

68_m. No acetamino-acid could be crystallized from this fraction. It is in this fraction that α-aminobutyric acid [cf. Vickery & Schmidt, 1931] would be expected to occur, if present (cf. Table 5). We conclude from the low N content of this fraction that α-aminobutyric acid is absent from, or at most a very minor constituent of, wool.

In this and all other 'M' fractions we obtained by crystallization from alcohol, followed sometimes by recrystallization from ether, small quantities (never more than 20 mg. from a fraction) of a substance having M.P. 120–121°. (Found: C, 35·2; H, 5·65; S, 59·8 %; N absent. $C_3H_6S_2$ requires C, 34·0; H, 5·67; S, 60·4 %.)

This substance was insoluble in water and extremely soluble in chloroform and ligroin. It was accompanied by a brown oil, which remained insoluble in water on boiling with mineral acid. The oil from fraction $68_c13_c7{\cdot}5_m$ after boiling with 2*N* HCl had S, 5·9 %.

A compound of the same composition and M.P., to which the formula $C_6H_{12}S_4$ was ascribed, was reported by Tucker & Reid [1932] to result in low yield from the interaction of CH_2Cl_2 with $CH_2SH.CH_2SH$ (formed by the interaction of H_2S with $CH_2Cl.CH_2Cl$). They postulated a cyclic polymethylene sulphide structure.

Although it is hard to see how the same compound could have resulted under the conditions employed by us, it seems reasonable to suppose that the present sulphur-rich bodies may be formed by the interaction of H_2S, etc. (resulting from the breakdown of cystine) with halogenated hydrocarbons. We are informed by Dr K. Bailey that 3–4 % of the S of wool is to be found in an acid hydrolysate as elementary S.

It is difficult to see why substances so soluble in chloroform and insoluble in water should remain in the column of the machine, rather than leave it in the chloroform effluent. Perhaps they are strongly adsorbed by the chloroform-water interface, the area of which is considerable while the machine is in operation.

$68_c13_w21_w$. In view of the high estimated methionine content of this fraction (19 % of N of fraction) it was subjected to immediate acid hydrolysis, with a view to carrying out the $HgCl_2$ precipitation described by Pirie [1932]. Possibly because of the small total quantity of material, this procedure did not give a satisfactory precipitation. The mixture was accordingly treated with H_2S to remove Hg, Ag_2O to remove Cl^- and H_2S to remove Ag. The filtrate from Ag_2S was evaporated to dryness. The crystalline residue was refluxed with alcohol, and after cooling was filtered off and washed with alcohol. The alcohol dissolved 216 mg. of non-volatile matter, which was not further investigated. The alcohol-insoluble material (308 mg.) had C, 47·7; H, 9·22; N, 7·55 %.

Some of this material was benzoylated by the method of Fischer [1900]. A 15 % yield of crystalline benzoylated product was obtained, which on recrystallization from water had M.P. 127°, not depressed by admixture with authentic benzoyl-*l*-valine, for which Karrer & van der Sluys Veer [1932] record M.P. 127°. Karrer & Schneider [1930] record benzoyl-*l*-*nor*valine as having M.P. 95°, and as forming a hydrate of M.P. 64°.

$68_c13_w21_m$. This fraction crystallized readily from a small volume of chloroform. The crystals, after filtering off and washing with chloroform, weighed 594 mg. and had M.P. 156°, which was not depressed on admixture with authentic acetyl-*l*-valine. $[\alpha]_D^{23°}+2{\cdot}5°$ (alcohol, $c=2$). (Synge [1939, 1] records acetyl-*l*-valine as having M.P. 157–158°. $[\alpha]_D^{20°}+5{\cdot}8°$.) (Found: C, 52·7; H, 7·84; N, 8·3 %. Calc. for $C_7H_{13}O_3N$: C, 52·8; H, 8·17; N, 8·8 %.) A further 19 mg. of the same material were obtained on crystallization of the material in the mother liquors from chloroform-ether. No further crystals resulting, the material in the mother liquors was hydrolysed with H_2SO_4, freed from $SO_4^=$ with $Ba(OH)_2$ and extracted with alcohol as described for the previous fraction. The resulting alcohol-insoluble material (104 mg.) had C, 50·4; H, 8·89; N, 9·3 %. Valine

$C_5H_{11}O_2N$ has C, 51·2; H, 9·41; N, 11·98%. A 1% solution in water showed no optical activity in a 2 dm. tube.

The alcohol extract yielded 302 mg. of non-volatile matter which did not crystallize readily, and has not been further investigated.

$68_c 13_w 21_c$. This fraction gave no crystals from chloroform, but crystallized slowly from moist ether. The product, on recrystallization from moist chloroform ligroin, weighed 532 mg. (air-dry). It had M.P. 77°, and did not depress the M.P. of authentic acetyl-*l*-proline hydrate (see under fraction $68_c 13_m$).

The material in the mother liquors was hydrolysed and extracted with alcohol as previously described. Only a very small amount remained insoluble in alcohol, and neither this nor the alcoholic extract has been further investigated.

$68_c 13_m$. This fraction yielded as crystals from chloroform a first crop of 473 mg., M.P. 183–184°, not depressed on admixture with authentic acetyl-*l*-leucine (cf. corresponding fraction from known mixture). $[\alpha]_D^{24°} -22·6°$ (alcohol, $c=3·3$). (Found: C, 55·0; H, 8·94; N, 7·64%. Calc. for $C_8H_{15}O_3N$: C, 55·5; H, 8·67; N, 8·1%.)

The mother liquors from this material, by successive crystallization from ligroin and moist ether-ligroin, yielded 615 mg. of material having M.P. 78° (115° after drying *in vacuo*). $[\alpha]_D^{22°} -114·0°$ (water, $c=4·2$) (rotation calculated for anhydrous compound). Du Vigneaud & Meyer [1932, 1, 2] record for acetyl-*l*-proline M.P. 81–82° (hydrate), 116–117° (anhydrous) $[\alpha]_D^{27°} -106·7$, $-107·1°$ (water, $c=0·5$). (Found: C, 52·9; H, 7·02; N, 8·62%. Calc. for $C_7H_{11}O_3N$: C, 53·5; H, 7·01; N, 8·91%.)

A second crop of the same material (M.P. 77°), weighing 203 mg., was obtained.

After this removal of acetylproline, it was possible to obtain a further crop of acetylleucine from a small volume of chloroform. 181 mg. $[\alpha]_D^{21°} -14·5°$ (alcohol, $c=1$). M.P. 155–169°. (Found: C, 55·5; H, 8·92; N, 6·4%.)

The small amount of material still remaining in the mother liquors has not been further investigated.

$68_c 13_c 7·5_w$. This fraction crystallized readily from water, and seemed nearly completely crystalline, although the later crops crystallized very slowly.

Crop I (852 mg.) had M.P. 175–179°. $[\alpha]_D^{20°} -13·0°$ (alcohol, $c=1·5$). (Found: C, 55·2; H, 8·63; N, 6·6%.)

Crop II (176 mg.) had M.P. 143–158°. $[\alpha]_D^{20°} -4·5°$ (alcohol, $c=1·3$). It contained 4% of incombustible residue, and after 'correction' for this, had elementary composition: C, 54·2; H, 8·13; N, 7·0%. The residue (275 mg.) has not been further investigated.

$68_c 13_c 7·5_m$. Crystallization from chloroform, which was very slow, yielded 180 mg. of material having M.P. 179–181° (crop I). $[\alpha]_D^{20°} -8·4°$ (alcohol, $c=1$). (Found: C, 54·8; H, 8·50; N, 7·6%.)

Crystallization of the residue in the mother liquor from chloroform-ligroin gave crystalline material melting at 133–148° (crop II). Repeated crystallization of this yielded crystals (34 mg.) having M.P. 153–163°. These were not further investigated. The remaining material of crop II had $[\alpha]_D^{20°} -1·7°$ (alcohol, $c=1$). (Found: C, 53·4; H, 7·87; N, 7·0%.)

The material in the mother liquors from crop II was hydrolysed with 2 *N* HCl at 100° for 3 hr., and on cooling the aqueous phase was separated from the oil (see under fraction 68_m) and evaporated to dryness several times with water. The residue was taken up in water, and brought to *p*H 6 with LiOH. The mixture was again evaporated to dryness, and extraction with alcohol yielded 38 mg. of insoluble material, which has not been further investigated.

68$_c$13$_c$7·5$_c$. This fraction did not readily give crystals from chloroform. Crystallization from ether-ligroin, followed by recrystallization from chloroform, gave 536 mg. of material having M.P. 141–143°, whose M.P. was not depressed on admixture with authentic acetyl-*dl*-phenylalanine of the same M.P. Despite reports in the literature of higher M.P. (151° [Knoop & Blanco, 1925], 150–151° [du Vigneaud & Meyer, 1932, 1], 148° [Neuberger, 1938, 2]), we agree with Jackson & Cahill [1938] in having been unable to prepare synthetic acetyl-*dl*-phenylalanine of M.P. higher than 143°. $[\alpha]_D^{20°}+2·4°$ (alcohol, $c=1·7$). For acetyl-*l*-phenylalanine Jackson & Cahill [1938] record $[\alpha]_D^{25°}+47·6°$ (alcohol), while for the *d*-isomer du Vigneaud & Meyer [1932, 1] record a corresponding figure of −51°. (Found: C, 63·1; H, 6·20; N, 5·9 %. Calc. for $C_{11}H_{13}O_3N$: C, 63·7; H, 6·28; N, 6·77 %.)

A further crop of 112 mg. of the same material was obtained.

As the residue would give no more crystals, it was hydrolysed and neutralized with LiOH in the same manner as the previous fraction. Precipitation by alcohol from concentrated aqueous solution yielded 144 mg. of material having $[\alpha]_D^{20°}$ −4·9° (water, $c=0·8$). (Found: C, 60·1; H, 8·01; N, 7·6 %.) This elementary composition is intermediate between that of phenylalanine and leucine (isomers) of which this material is presumably a mixture.

Isolation of hydroxyamino-acid fraction from wool hydrolysate

The aqueous solution resulting from the second extraction of the acetylated hydrolysate in the Neuberger apparatus was evaporated to dryness and the acetamino-acids were freed from salt by extraction with alcohol [cf. Synge, 1939, 2]. The product resulting from evaporation of the alcohol was refluxed for 4 hr. with 450 ml. of 3*N* H_2SO_4. This solution, when cool, was treated with 75 g. of phosphotungstic acid at a final volume of a litre. After storing for 15 hr. the precipitate was filtered off, and washed with a 1 % solution of phosphotungstic acid in 1 % H_2SO_4. The precipitate was found (by difference) to contain 29·3 % of the total protein-N.

The combined filtrate and washings from this were treated with $Ba(OH)_2$ at 100° until just permanently alkaline to thymol blue. The resulting precipitate was thoroughly extracted with hot water, being maintained at the same *p*H by addition of $Ba(OH)_2$ when necessary; the filtrate and washings were then combined, and concentrated to a medium syrup. This was subjected to the acetylation-benzoylation procedure described by Synge [1939, 3], employing for the acetylation 200 ml. of 2*N* NaOH and 20 ml. of acetic anhydride, and for the benzoylation 185 ml. of 2*N* NaOH and 23 ml. of benzoyl chloride.

After the saponification of the *O*-benzoyl groups by $Ba(OH)_2$ as described Ba was removed exactly before the chloroform extraction, after which the aqueous layer was concentrated to dryness, giving the hydroxyamino-acids as their *N*-acetyl derivatives.

This fraction contained 5·9 % of the protein-N (5·6 % amino-N), in good agreement with the results obtained on wool by Synge [1939, 3] without previous removal of the higher monoamino-acids. The *N*-acetyl-hydroxyamino-acids formed a nearly colourless glass, which showed no sign of crystallization after storage in a desiccator for 6 months.

In a forthcoming paper we describe the further investigation of this fraction.

Isolation of the hydroxyamino-acids by acetylation-benzoylation after removal of the higher monoamino-acids by extraction of their acetyl derivatives into chloroform appears to be preferable to the direct procedure, since it minimizes the possibility of contamination of the fraction by monoacetamino-

acids, which pass readily into chloroform from strong salt solution during the extraction of the *N*-acetyl-*O*-benzoyl-hydroxyamino-acids, but may not be so readily extractable by chloroform from the salt-free solution resulting from the saponification of the *O*-benzoyl derivatives.

Discussion

The valine fractions. Abderhalden & co-workers [1930–37] have reported the occurrence of *l-nor*valine with valine in globin, casein, tusscre silk, steer-horn and yeast proteins. We have obtained no positive evidence for its occurrence in wool, about two-thirds of the possible valines in fractions $68_c13_w21_w$ and $68_c13_w21_m$ having been identified as *l*-valine in the form of its acetyl and benzoyl derivatives.

The leucine fractions. The rotations and M.P. of the various preparations isolated having the composition of acetylleucine are summarized in Table 14.

Table 14. *Acetylleucine isomers from wool*

Fraction	Crop	Wt. (mg.)	M.P.	$[\alpha]_D$ (alcohol)
68_c13_m	I	473	183–184°	−22·6°
	II	181	155–169°	−14·5°
$68_c13_c7{\cdot}5_w$	I	852	175–179°	−13·0°
	II	176	143–158°	− 4·5°
$68_c13_c7{\cdot}5_m$	I	180	179–181°	− 8·4°
	II	34	153–163°	—
	III	241	133–148°	− 1·7°

From the data provided by Synge [1939, 1] it may be inferred that acetyl-*l*-leucine has M.P. 186–188°, $[\alpha]_D-23{\cdot}2°$ (alcohol), and acetyl-*l-iso*leucine, M.P. 150–151°, $[\alpha]_D+15{\cdot}6°$ (alcohol).

Acetyl-*l*-leucine does not on admixture depress the M.P. of acetyl-*l-iso*leucine or of acetyl-*dl*-leucine. Acetyl-*dl*-leucine on admixture depresses the M.P. of acetyl-*l-iso*leucine. It seems therefore unnecessary to postulate the occurrence of *nor*leucine in wool in order to account for the M.P. and rotations of the acetyl-leucine fractions isolated. Acetyl-*d-nor*leucine [Synge, 1939, 1] has the low M.P. 112–114° and an insignificant rotation in alcohol.

It has been shown in the control experiment described above that the procedure employed racemizes leucine to the extent of about 15%. In the case of *l-iso*leucine, a corresponding epimerization to *d-alloiso*leucine may be expected. Since *d-alloiso*leucine has $[\alpha]_D-14{\cdot}2°$ (water), $-36{\cdot}8°$ (20% HCl) [Ehrlich, 1907], which is nearly equal and opposite to that of *l-iso*leucine ($[\alpha]_D+11{\cdot}3°$ (water), $+37{\cdot}4°$ (20% HCl) [Locquin, 1907]), it seems likely that the same may be true for the corresponding acetyl derivatives. Assuming this to be the case, and that 15% racemization (epimerization) has occurred, the calculated leucine : *iso*-leucine ratio in the isolated crops of acetylleucine isomers would be 4 : 1. The proportion of *iso*leucine left in the mother liquors would be greater than this, on account of the greater solubility of its acetyl derivative.

The phenylalanine fraction. No isolation of phenylalanine has been previously reported from wool, although Block [1939] reports a colorimetric determination, giving the phenylalanine-N as 2·2% of the total N of the wool. Abderhalden & Voitinovici [1907] obtained in the ester fraction in which phenylalanine was to be expected a product having the elementary composition of leucine. In the present work we have isolated 1·32% of the N of wool as a product agreeing in its properties with acetyl-*dl*-phenylalanine and having only

4·7 % of the optical activity of acetyl-*l*-phenylalanine. Moreover, since acetyl-*l*-phenylalanine is less soluble and crystallizes more readily than the racemic compound, there is no reason to suspect significant quantities of it in the mother liquors. The possibility of extensive racemization in the course of acetylation may be ruled out, since the procedure employed gave, in the hands of du Vigneaud & Meyer [1932, 1], a 92 % yield of acetyl-*l*-phenylalanine from *l*-phenylalanine, and we can confirm this from our own experience. Moreover, the other amino-acids in the same hydrolysate were not seriously racemized.

It does not seem to us necessary to postulate the occurrence of phenylalanine residues in wool other than in the *l* form. Levene & Steiger [1930] have shown that treatment of *d*-phenylalanine for 36 hr. at 100° with 20 % HCl induces no detectable racemization, whereas in glycyl-*d*-phenylalanine and the corresponding diketopiperazine up to 20 % racemization of the phenylalanine was observed under the same conditions. Little, if any, other work has been published on the racemization of amino-acid residues during the treatment of peptides etc. with mineral acids, although the question has assumed importance in connexion with the recent controversy over the occurrence of partially racemic glutamic acid in tumour hydrolysates. The earlier stages of this controversy have been reviewed by Pirie [1939; cf. also Graff *et al.* 1940, and Chibnall *et al.* 1940]. These last workers observe: 'Protein chemists have so far given but little attention to the occurrence of racemized amino-acids in protein hydrolysates, and it may well be that they are more widespread than has been imagined, for the possibility that racemization accompanies the splitting of certain peptide bonds during hydrolysis of the protein molecule must not be lost sight of.'

It seems to us possible that the position occupied by the phenylalanine residues in wool may be such as to render them more liable than other residues to racemization in the course of acid hydrolysis. In this connexion it is interesting that Jensen & Evans [1935], by subjecting insulin to treatment with phenyl*iso*-cyanate, followed by acid hydrolysis, isolated a yield of the phenylhydantoin of *dl*-phenylalanine which suggested that a substantial proportion of the amino groups of this protein belong to phenylalanine residues.

It is possible that the present phenomenon may not be restricted to wool: Sharp [1939] isolated very considerably racemized phenylalanine from a myosin hydrolysate by ester distillation, and Baptist & Robson [1940] have commented on the high degree of racemization recorded in the literature for phenylalanine preparations from proteins.

Summary of present analysis

In Table 15 we summarize our analytical data on the seven end-fractions which we have investigated. In this table, each amino-acid is expressed in N as % of total N of the wool. Under each amino-acid col. I records material isolated and formally identified, while col. II refers to 'unidentified N'. In the cases of methionine and proline, these figures are based respectively on the 'volatile iodide' and 'non-amino-N' figures for each fraction. In the cases of valine, leucine isomers and phenylalanine, the figures in col. II are based on the residual amino-N of the fraction, and are allocated, in the case of fraction $68_c 13_c 7{\cdot}5_c$, on the basis of the elementary composition of the material isolated by acid hydrolysis from the mother liquors of acetylphenylalanine.

The total N of the end-fractions (17·87 % of total N) is in good agreement with the N figure for fraction 68_c (18·2 %), showing that the loss of material in the course of analysis is slight.

Table 15. *The higher monoamino-acids of wool*

(N as % of total N)

Fraction	N of fraction	Methionine I	Methionine II	Valine I	Valine II	Proline I	Proline II	Leucines I	Leucines II	Phenylalanine I	Phenylalanine II
$68_c13_w21_w$	1·66	—	0·31	0·17	0·92	—	0·26	—	—	—	—
$68_c13_w21_m$	2·81	—	0·09	2·01	0·16	—	0·55	—	—	—	—
$68_c13_w21_c$	1·72	—	0·01	—	0·13	1·29	0·16	—	0·13	—	—
68_c13_m	4·44	—	—	—	—	1·97	0·70	1·60	0·17	—	—
$68_c13_c7{\cdot}5_w$	3·17	—	—	—	—	—	—	2·52	0·65	—	—
$68_c13_c7{\cdot}5_m$	1·55	—	—	—	—	—	—	1·11	0·44	—	—
$68_c13_c7{\cdot}5_c$	2·52	—	—	—	—	—	—	—	0·58	1·32	0·62
Total	17·87	—	0·41	2·18	1·21	3·26	1·67	5·23	1·97	1·32	0·62
Combined totals			0·41		3·39		4·93		7·20		1·94

In Table 16 the present analysis is correlated with the more important published data on the amino-acid composition of wool. In this table the amount of each amino-acid is expressed as N in % of total N of wool. Where authors have not recorded the N content of the wool analysed, it has been assumed to be 16·75 %. Barritt [1928] found the N content of a very wide variety of white wools to lie between 16·50 and 17·07 %.

The high amino-N content of the hydroxyamino-acid fraction from wool makes the presence of hydroxyproline unlikely.

This is in agreement with the failure of Abderhalden & Voitinovici [1907] to isolate hydroxyproline from wool or from sheep-horn where they made a special search for it.

Table 16. *Summarized data on the amino-acid composition of wool*

(N as % of total N)

Author ...	Abderhalden & Voitinovici [1907]	Marston [1928]	Block [1939]	Other authors	Present investigation
N content of wool %	—	17·8	15·4	—	—
Glycine	0·65	—	7·87	—	—
Alanine	4·13	—	—	—	—
Valine	2·0	—	—	—	3·4
Leucine isomers	7·35	—	—	—	7·2
Phenylalanine	—	—	2·2	—	1·9
Proline	3·20	—	—	—	4·9
Methionine	—	—	—	0·3 Barritt [1934]	0·4
Cystine	5·08	8·59	9·93	—	—
Serine, etc.	0·05	—	—	7·5 Martin & Synge	—
Threonine	—	—	—	4·7 (to be published)	—
Tyrosine	1·34	2·08	2·26	—	—
Tryptophane	—	1·39	0·62	—	—
Aspartic acid	1·45	—	—	4·56 Speakman &	—
Glutamic acid	7·34	—	—	8·68 Townend	—
Ammonia	—	6·74	—	8·18 [1937]	—
Arginine	—	18·42	18·16	20·2 Vickery [1940]	—
Histidine	—	10·50	1·23	—	—
Lysine	—	5·02	3·12	—	—

It will be seen from Table 16 that more than 90 % of the N of wool has now been reasonably accounted for in the form of ultimate hydrolytic products. The corresponding figure for gelatin, based on the figures of Dakin [1920], as modified by Bergmann & Stein [1939] for glycine and proline and by Synge [1939, 3] for hydroxyamino-acids other than hydroxyproline, is 92·5 %.

The serine and threonine values given in Table 16 are taken from our forthcoming paper.

Criticism of the new analytical procedure

In appraising the analytical value of the technique described in this paper, we wish to emphasize that the procedure is still in a crude state and is capable of technical development. Thus it is clear, from the consistent change of rotation of successive acetylleucine fractions shown in Table 14, that a longer extraction column, operating in a more controlled manner, could give practically complete separation of the components of this mixture.

It is natural to compare the present procedure with the Dakin butyl alcohol extraction and Fischer ester distillation procedures, to which its two stages are directly analogous, and which are the only methods for separating this group of amino-acids in general use among protein chemists.

The present procedure is not suited to deal with glycine or alanine. For methionine, valine, proline, leucine isomers and phenylalanine it has definite advantages. The procedure leading to fraction 68_c gives these amino-acids in 95% yield, and substantially free from amino-acids of other groups. Butyl alcohol extraction, according to Sharp [1939], may give significant amounts of basic and dicarboxylic amino-acids together with the monoamino-acids. In subsequent fractionation, the present procedure leads to a strikingly better separation of valine from leucine than those recorded for ester distillation in the literature. It should, however, be remembered that modern distillation technique has so far not been applied to the Fischer distillation. The degrees of racemization induced by the two procedures seem to be similar. The acetamino-acids have the advantage over the free amino-acids of a lower tendency to form mixed crystals, and therefore may prove of use for 'isotope dilution' analysis [Rittenberg & Foster, 1940] where high purity is called for, irrespective of yield. The acetamino-acids have definite melting points, which is useful for rapid identification. The quantity of protein required for an analysis (10 g.) by the present procedure is much smaller than that required for an ester distillation. Finally, the residues from the first extractions may advantageously be used for the isolation of hydroxyamino-acids by acetylation-benzoylation, as described above, and it is possible that other analytical procedures would benefit from a preliminary removal of the higher monoamino-acids.

Summary

1. The history of the development of purification processes based on liquid-liquid extraction is reviewed.

2. The possibility of separating certain amino-acids as their acetyl derivatives by extractional fractionation in a chloroform-water counter-current train is discussed. The physical chemistry of the separation is outlined, and some new experimental data are provided on the equilibria concerned.

3. The design, construction and operation of a 40-unit counter-current liquid-liquid extraction train is described, together with tests of the efficiency of this machine in separating acetamino-acids.

4. On this basis an analytical procedure for the determination and isolation of methionine, valine, proline, leucine isomers and phenylalanine in protein hydrolysates is developed, and is tested on a complex known mixture of amino-acids. The results show that the new procedure has some advantages over the use of butyl alcohol extraction (Dakin) followed by ester distillation (Fischer).

5. The new procedure is applied to the analysis of a wool hydrolysate.

6. The results of the present investigation are summarized together with the published information on the amino-acid composition of wool.

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13. ENZYMIC PROTEOLYSIS

4. AMINO-ACIDS OF CASEIN PHOSPHOPEPTONE[1]

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Trypsin-resistant phosphopeptones from casein have been studied by Posternak [1927; 1928] and by Rimington [1927, 1, 2]. The former isolated three compounds which he designated 'lactotyrins' and showed them to be made up of 15 to 18 amino-acid units containing serine, glutamic acid, aspartic acid and *iso*leucine. Posternak's papers contain no experimental details, and although his conclusion that the P in casein exists in the form of phosphoserine has been confirmed by the isolation of this amino-acid by Lipmann [1933], the generally accepted view of the structure of phosphopeptone itself is that advanced by Rimington [cf. Jordan Lloyd & Shore, 1938], according to whom the enzyme-resistant polypeptide is made up of 9 hydroxyamino-acid units consisting of 3 mol. of hydroxyglutamic acid, 4 mol. of hydroxyaminobutyric acid and 2 mol. of serine. The discrepancies in the results obtained by these authors and the fact that Harington & Randall [1931] were unable to identify hydroxyglutamic acid as a constituent of casein prompted the reinvestigation of this problem.

To obtain a trypsin-resistant phosphopeptone of constant composition advantage was taken of the fact that peptic digestion of casein automatically causes the separation of an insoluble complex, the so-called 'paranuclein', which contains 50–60 % of the P of casein and only about 20 % of its N. After digesting the paranuclein till constancy of amino-N was reached, the P-containing complex was precipitated with neutral lead acetate, redigested with trypsin and the phosphopeptone finally separated as the Ba salt insoluble in 50 % alcohol. Preparations so obtained were constant in composition and unaffected by further treatment with trypsin. The amino-N value of 10 % and the atomic N/P ratio of 3·2 to 3·3 indicated a polypeptide of 10 amino-acids attached to 3 phosphoric acid residues. N distribution determined according to Damodaran [1931, 1] gave amide-N values varying from 10 to 13 % in different analyses, dicarboxylic acid-N 43–47 % and monoamino-N 43–45 %, and a complete absence of basic and non-amino-acids. For isolation of the amino-acids present a combination of the butyl alcohol [Dakin, 1918], the Ba-alcohol [Kingston & Schryver, 1924] and the copper salt [Damodaran, 1931, 2] methods were used. Serine, *iso*leucine and glutamic acid were found to be the only amino-acids present. It was also seen early in the investigation that the glutamic acid isolated accounted for less than two-thirds of the dicarboxylic acid-N as found by N-distribution. In view of the previously reported presence in phosphopeptone of dicarboxylic acids other than glutamic acid and of hydroxyamino-acids other than serine, it was decided to determine hydroxyamino-acids and dicarboxylic acids by indirect methods in all amino-acid fractions separated from the hydrolysate.

For the identification of the hydroxyamino-acids oxidation with chloramine-T and subsequent preparation of suitable osazones of the hydroxy-aldehydes

[1] First appeared as a note in *Nature, Lond.*, **145**, 857

formed [Dakin, 1916; 1917] was made use of. The *p*-nitrophenyl- and the 2:4-dinitrophenyl-osazones of glyoxal (from serine), of methylglyoxal (from hydroxyaminobutyric acid) and of malic semi-aldehyde (from hydroxyglutamic acid) have M.P. ranging between 299 and 319° and are therefore unsuited for identification; the phenylosazones, on the other hand, of methylglyoxal and glyoxal, though less insoluble, have M.P. 145 and 167° respectively, which make them useful for differentiation. Figures are not available for the M.P. of the phenyl- and the 2:4-dinitrophenyl-osazones of malic semialdehyde. By the use of these derivatives it was found that there was no evidence for the presence of any hydroxyamino-acid other than serine. As quantitative isolation of this amino-acid was naturally difficult the insolubility of the 2:4-dinitrophenyl-osazone formed from it was made use of for quantitative estimation.

For the indirect estimation of dicarboxylic acids at various stages of separation the titration method of Harris [1929] was used. It soon became obvious that the amount of glutamic acid isolated was much less than the amount indicated either by the Foreman method or by titration. This discrepancy, which could not be accounted for by losses in isolation, was finally found to be caused in the case of the Foreman method by the precipitation of serine along with glutamic acid and in the titration method by the presence of ether-extractable non-nitrogenous fatty acids. From the precipitate obtained by treatment with $Ca(OH)_2$ or $Ba(OH)_2$ and alcohol according to Foreman glutamic acid and serine could be separated by fractional crystallization. Further, working with pure serine it was found that from a 3·3% solution treated by this method this amino-acid is precipitated to the extent of 63·8%. In the titration method the high values for dicarboxylic acid obtained were eliminated if the hydrolysate were first submitted to prolonged extraction with ether, the residual titratable acidity being then equivalent to the glutamic acid that could be isolated.

The ether-extractable acidic material, which was free from nitrogen, was found to be a mixture, the main component of which was glyceric acid, the latter being isolated and identified as its quinine salt. Varying amounts of lactic and pyruvic acids were also usually present, the quantities of the two being determined by the methods of Leib & Zacherl [1932] and of Simon & Neuberg [1931] respectively. The nature of the non-nitrogenous fatty acids indicated their secondary origin in the decomposition of serine [Abderhalden & Broich, 1933], and suggested that the NH_3 formed during acid hydrolysis of the phosphopeptone also had the same origin. The amide-N did in fact vary in different analyses; on following the time course of NH_3 liberation during hydrolysis by dilute acid according to Vickery [1922] it was seen that the NH_3 did not arise from an amide group but from deamination. The equivalence of the ether-extractable acidity to the NH_3 present in any particular hydrolysate left no doubt that the NH_3 was formed by the secondary decomposition of serine.

The results of the analyses by direct and indirect methods are summarized in Table 1.

Table 1. *Amino-acid composition of phosphopeptone*

	N as % of total N	
Amino-acid	By isolation	By estimation
Glutamic acid	29·70	29·97
*iso*Leucine	27·90	—
Serine	13·10	28·90
Ammonia	—	10·90

The hydrolysate is seen to consist of 30 % each of the three amino-acids, serine, glutamic acid and *iso*leucine. Assuming, as we are justified in doing, that the NH_3 formed during hydrolysis represents decomposed serine, it is concluded that the phosphopeptone is a polypeptide of 10 amino-acids made up of 4 mol. of serine and 3 mol. each of glutamic acid and *iso*leucine.

Experimental

Materials and methods

Casein was prepared (from reconstituted milk made with Australian skimmed milk powder) by the method of Cohn & Hendry [1930] and contained 15·6 % N and 0·8 % P. B.D.H. pepsin and trypsin were used for digestion.

The following methods were frequently made use of:

Oxidation of the fractions with chloramine-T and preparation of the osazones	Dakin [1918] and Gulland & Morris [1934] with modifications
Titration of dicarboxylic amino-acids	Harris [1929]
Determination of lactic acid	Leib & Zacherl [1932]
Determination of pyruvic acid	Simon & Neuberg [1931]
Organic and inorganic P	Fiske & Subbarow [1925]

Preparation of enzyme-resistant phosphopeptone

(*a*) *Preparation of 'paranuclein'*. 5 kg. of wet freshly precipitated casein, corresponding to about 1 kg. dry weight, were uniformly dispersed in 15 l. of $N/10$ H_2SO_4 at 37°, the *p*H adjusted to 1·8 by the addition of 5 N H_2SO_4 and the mixture incubated under toluene after addition of 25 g. of pepsin dissolved in 100 ml. of $N/10$ H_2SO_4. After 48 hr. the *p*H, which had risen to 2·2, was brought back to 1·8 and a further 25 g. of pepsin added. Amino-N reached constancy at about 20 % in 7 days. No inorganic P was formed at this stage in the digest. From the paranuclein formed as a compact precipitate at the bottom most of the clear yellow supernatant liquid was siphoned off and the remaining suspension was poured into large fluted filters and allowed to drain overnight in the refrigerator. The pasty solid was collected, stirred up with large volumes of water and washed free from H_2SO_4 by decantation and filtration as before. The product obtained after drying contained 15·2–15·6 % N, about 2 % P and had an atomic N/P ratio of 15–18 (compared with a ratio of about 44 for casein). Amino-N by the Van Slyke method was 12 %. The usual yield of dry paranuclein from 1 kg. of casein was 200–220 g., representing about 20 % of the N and 50–60 % of the P of casein.

(*b*) *Digestion of paranuclein with trypsin*. Trial experiments showed that digestion with trypsin at *p*H 8·3 caused only minimal liberation of inorganic P. At the end of 7 days when tryptic hydrolysis had reached completion (at 50–52 % of total hydrolysis) the inorganic P present was less than 5 %. The wet paranuclein from the peptic digestion was suspended in about 3 l. of distilled water and N NaOH added slowly with vigorous mechanical stirring till solution was complete. The solution, the *p*H of which was usually about 7 at this stage, was brought to 8·3 by the further addition of NaOH and incubated at 37° under toluene with 5 g. of trypsin. After 24 hr. amino-N of the solution was 40 % of the total N and the *p*H 6·3. No inorganic P was present. After readjusting the *p*H and adding a further 5 g. of trypsin, digestion was allowed to continue till amino-N failed to increase. This happened usually in 7 days when amino-N was about 53 % and about 5 % of the P was present in inorganic condition.

(*c*) *Precipitation of phosphopeptone as Ba salt.* The digest was now brought to *p*H 4·6 with glacial acetic acid and after keeping overnight in the ice-chest was filtered from the small amount of suspended material. To the filtrate a concentrated solution of neutral Pb acetate was added with vigorous stirring till precipitation was complete. The Pb precipitate, which contained the whole of the organic P of the digest, was separated by centrifuging, suspended in water and decomposed by H_2S. The filtrate (about 1·5 l.), freed from Pb and H_2S, was redigested with 2 g. of trypsin and reprecipitated with Pb acetate as before. The solution obtained after decomposing the precipitate and freeing from H_2S was treated with 30 g. of Ba acetate, made alkaline with $Ba(OH)_2$ and rapidly filtered into a beaker containing acetic acid. After adjusting the *p*H to 7 by the addition of $Ba(OH)_2$ an equal volume of alcohol was added with stirring. The fine precipitate obtained was separated on the centrifuge, washed repeatedly with 50 % alcohol, then with absolute alcohol and ether and finally dried *in vacuo* over H_2SO_4.

Redigestion of the Pb precipitate with trypsin was found to be an essential condition for obtaining a Ba salt of constant composition which was resistant to further enzyme action. In earlier experiments in which the decomposed Pb salt was directly converted into the Ba compound, amino-N values ranging from 7·96 to 9·72 % and N/P ratios varying from 2·6 to 3·72 were obtained. However, preparations obtained as described above showed little variation in composition, as will be seen from the analyses of different samples (Table 2).

Table 2. *Analyses of Ba phosphopeptones*

Preparation no.	Amino-N as % of total N	N %	P %	N/P atomic	Ba %	Opt. rotation $[\alpha]_D^{31°}$ in H_2O
III	9·94	6·48	4·53	3·20	33·45	+34·81°
IV	9·84	6·44	4·26	3·35	33·12	
V	9·98	6·39	4·58	3·16	34·02	
VI	9·96	6·46	4·34	3·30	33·31	

Properties of the phosphopeptone

The Ba salt was extremely soluble in water, giving a water-clear solution which was neutral in reaction. The free peptide obtained by removing the Ba quantitatively with H_2SO_4 was strongly acidic. It was not precipitable by trichloroacetic, phosphotungstic or tannic acid, but was precipitated by heavy metal salts such as Pb, Cu and U acetates. It gave none of the usual colour reactions for individual amino-acids and was free from inorganic P. It underwent no hydrolysis on treatment with trypsin or intestinal erepsin. The whole of the P was split off by 1 % alkali in 24 hr. When an aqueous solution, made alkaline with $Ba(OH)_2$, was kept at 37° for a few hours and filtered from the precipitated Ba phosphate, the filtrate after being quantitatively freed from Ba was slowly acted upon by trypsin, amino-N increasing from 10 to 25 % in the course of a week. The resistance of the phosphopeptone to enzyme action is thus obviously due to the presence of the phosphoric acid groups.

Nitrogen distribution

N-distribution was determined according to Damodaran [1931, 1]. The results are given in Table 3.

Table 3. *Distribution of N in the phosphopeptone*

	Nitrogen	
	mg.	%
Total N	171·10	100·00
Humin-N	0·00	0·00
Ammonia-N	18·65	10·90
Dicarboxylic acid-N	77·59	45·37
Monoamino-N	73·41	42·90
Basic-N	0·00	0·00
Non-amino-N	0·00	0·00
Total	169·65	99·17

Separation of amino-acids in the phosphopeptone (scheme I)

Outline. The phosphopeptone was hydrolysed with H_2SO_4 and the hydrolysate (A), after being freed from H_2SO_4, phosphoric acid and NH_3, was treated with $Ba(OH)_2$ and alcohol. From the precipitate (B), after removal of Ba quantitatively, glutamic acid (Bi) and serine (Bii*a*) were obtained by fractional crystallization. A small amount of uncrystallizable syrup was left. The filtrate (C) from the Ba-alcohol precipitate, after being freed from Ba quantitatively, gave on fractional crystallization two or three crops of *iso*leucine (Ci) and finally serine (Cii*a*).

In all the fractions serine was determined by conversion into the dinitrophenylosazone of glyoxal and the apparent dicarboxylic acid content by titration according to the method described by Harris [1929] for the determination of unbalanced carboxyl groups in dicarboxylic monoamino-acids.

Hydrolysis. 15 g. of the Ba salt of the phosphopeptone were dissolved in 37·5 ml. of water, 14 ml. of conc. H_2SO_4 added and the mixture boiled under reflux for 18 hr. After making up to volume and removing an aliquot for analysis, H_2SO_4 and phosphoric acid were removed by means of $Ba(OH)_2$, the former at neutrality to Congo red and the latter at strongly alkaline reaction. The $BaSO_4$ and the Ba phosphate contained respectively 5·23 and 1·0 % of the total N. The alkaline filtrate from the Ba phosphate was concentrated *in vacuo* at 40° to about 1/5 vol. (about 20 ml.) for the removal of NH_3 and treated with 200 ml. of alcohol little by little with vigorous shaking to precipitate the dicarboxylic acids. The mixture was centrifuged and the sticky precipitate washed twice with 20 ml. portions of 95 % alcohol. The precipitate was dissolved in 20 ml. of hot water and after adding a small amount of $Ba(OH)_2$ was reprecipitated by the addition of 200 ml. of alcohol. The precipitate (B) was dissolved in water and made up to volume. It contained 47·7 % of the total N of the hydrolysate. The filtrate (C) from the Ba-alcohol precipitate was freed from alcohol by evaporation *in vacuo* and worked up as described later.

Dicarboxylic acid fraction (B). The solution was quantitatively freed from Ba, the $BaSO_4$ thoroughly washed with boiling water and the washings added to the main fraction. A portion of the solution was oxidized with chloramine-T and the serine-N was found to be 33·18 % of the N of this fraction. Titration of another portion of the solution showed an acidity corresponding to 100·89 % of dicarboxylic acid-N. The main solution was concentrated in the desiccator when thick prismatic crystals were obtained. The first crop weighed 1·8 g., while a second crop of 0·5 g. was obtained after further concentration. The N in these fractions amounted to 29·7 % of the total N of the hydrolysate and 63 % of the N of the Ba-alcohol precipitate. The two crops were combined (Bi) and recrystallized from water and the substance was identified as glutamic acid having N 9·53 %,

amino-N 9·57 % (for glutamic acid, N 9·56 %) and M.P. 199°, which was not lowered by admixture with pure glutamic acid. 41 mg. of the acid required 2·82 ml. of 0·98 *N*/10 NaOH for neutralization to bromothymol blue, giving mol. wt. 148·4 (calc. 147·0). By saturating a concentrated solution of the substance with HCl gas in the cold it was quantitatively converted into glutamic acid hydrochloride with M.P. 213° and N 7·57 % (calc. for glutamic acid hydrochloride, N 7·63 %, M.P. 214°).

The mother liquor from the glutamic acid (fraction Bii) contained 0·1322 g. of N, of which 88 % was serine-N, and had a titratable acidity corresponding to 109 % of dicarboxylic acid-N. By treatment with alcohol as described in detail under the monocarboxylic acid fraction (C), 0·1 g. of crystalline amino-acid was obtained (Bii*a*). The needle-shaped crystals on recrystallization from aqueous alcohol yielded beautiful hexagonal plates melting at 228° and containing 13·23 % N (for serine, N 13·33 %, M.P. 228°). For further identification a portion of the substance was oxidized with chloramine-T; the resulting solution was divided into three portions and treated respectively with phenylhydrazine, *p*-nitrophenylhydrazine and 2:4-dinitrophenylhydrazine. The osazones obtained were recrystallized, the phenylosazone from aqueous alcohol and the nitrophenylosazones from hot nitrobenzene. A portion of serine was similarly oxidized and the osazones were prepared. The M.P. and the mixed M.P. are given in Table 4.

Table 4. *Melting points of osazones*

	M.P. of osazones		
	From Bii*a*	From serine	Mixed
Phenylosazone	167°	167·5°	167°
p-Nitrophenylosazone	311°	311°	311°
2:4-Dinitrophenylosazone	318°	319°	318°

The uncrystallizable mother liquor (Bii*b* containing 0·1109 g. N) contained 84·7 % of serine as determined by oxidation and was also strongly acidic. But persistent attempts failed to show the presence of a dicarboxylic acid in this fraction.

Monocarboxylic acid fraction (*C*). The filtrate from the Ba-alcohol precipitate contained 0·3054 g. N or 40·44 % of the total N of the hydrolysate. The solution was freed from Ba quantitatively and allowed to concentrate in the desiccator. A white amorphous material separated on the sides of the dish and on the surface of the liquid. The material was broken up and filtered and the mother liquor on further concentration gave two more crops of a similar substance. The N contents were 10·63, 10·68 and 10·98 % respectively. The first crop did not contain any serine-N, while the other two crops contained 3 and 10 % serine-N respectively. The crops were mixed together (Ci) and recrystallized from water in which they were only sparingly soluble, when the same amorphous material as before was obtained (1·54 g.) but without contamination with serine. When the substance was dissolved in a small amount of hot water and alcohol was added to turbidity, needle-shaped crystals aggregated in bunches were obtained. The M.P. of the original amorphous material was 265°, while the crystals melted at 275°. The N content of 10·68 % indicated that the substance was one of the leucines.

For identification the substance was converted into its Cu salt. 1 g. of the material was dissolved in a small amount of water on the water bath and copper carbonate was added little by little till no more dissolved. The mixture was heated for about 30 min. on the water bath and filtered. The residue of copper

carbonate was thoroughly washed with hot water till the washings were colourless. The combined filtrate and washings were evaporated to dryness on the water bath and the powder dried to constant weight at 100° in the air oven. It was repeatedly extracted by shaking with dry methanol freshly distilled over Ca, when almost the whole of the substance went into solution leaving a negligible residue. The methanol-soluble Cu salt was freed from alcohol by evaporation and recrystallized from water, from which it was obtained in the form of light blue needles. The crystals contained N 8·63 and Cu 19·63 % (calc. for Cu *iso*leucine, N 8·47 and Cu 19·51 %). A small amount of the Cu salt was decomposed by H_2S and the solution on concentration gave beautiful hexagonal plates of *iso*leucine melting at 275°. A 3·42 % solution of the substance in 20 % HCl had $[\alpha]_D^{31°} + 26·5°$.

The mother liquor from Ci was heated on the water bath and alcohol was added drop by drop to turbidity. The sides of the dish were well scratched and the dish was left in the ice-chest overnight, when a mass of needle-shaped crystals was obtained. These were filtered quickly under centrifugal force, washed with alcohol and dried (Cii*a*). Another crop was obtained by concentration and addition of alcohol as before. The combined crops (0·448 g.) were recrystallized from aqueous alcohol. The hexagonal plates resembled fraction Bii*a* closely in all respects: N 13·32 %, M.P. 228°. The osazones were also identical with those of Bii*a*. The substance was therefore serine.

Table 5. *Analyses of fractions in scheme I*

	Fractions	N of fractions		
		In g.	As % of N of fraction	As % of T.N. of hydrolysate
A.	Hydrolysate	0·7554	—	100·00
	Dicarb.-N	0·3358	—	44·46
	Serine-N	0·2099	—	27·80
	Ammonia-N	0·0825	—	10·92
B.	Ba-alcohol precipitate	0·3603	—	47·70
	Dicarb.-N	—	100·89	—
	Serine-N	—	33·18	—
Bi.	*Glutamic acid* (2·3 g.)	0·2244	—	29·70
Bii.	Filtrate from (Bi)	0·1322	—	17·50
	Dicarb.-N	—	109·30	—
	Serine-N	—	88·00	—
Bii*a*.	*Serine* (0·1 g.)	0·0132	—	1·75
Bii*b*.	Mother liquor from above: uncrystallizable	0·1109	—	14·67
	Dicarb.-N	—	110·00	—
	Serine-N	—	84·72	—
C.	Filtrate from Ba-alcohol precipitate	0·3054	—	40·44
	Dicarb.-N	—	0·00	—
	Serine-N	—	34·72	—
Ci.	iso*Leucine* (1·54 g.)	0·1644	—	21·78
Cii.	Filtrate from above	0·1280	—	16·94
	Dicarb.-N	—	0·00	—
	Serine-N	—	82·56	—
Cii*a*.	*Serine* (0·45 g.)	0·0585	—	7·75
Cii*b*.	Uncrystallizable mother liquor	0·0629	—	8·33
	Dicarb.-N	—	0·00	—
	Serine-N	—	76·23	—

No crystals were obtained from the mother liquors B ii*b* and C ii*b* left from the dicarboxylic and the monocarboxylic acid fractions respectively. The total N in the fractions amounted to about 23 % of the hydrolysate-N. The fractions consisted mainly of serine, the amounts of which in the two fractions were 84·7 and 76·2 % respectively. They probably contained small amounts of the other amino-acids and also serine-decomposition products which prevented the amino-acids from crystallizing. On oxidation of the fractions only glyoxal osazones were obtained, which showed that serine was the only hydroxyamino-acid present in the hydrolysate. Conversion into Cu salts failed to show the presence of any aspartic acid. The analyses of the various fractions are given in Table 5.

Separation of the amino-acids in the phosphopeptone (*scheme II*)

Outline. The hydrolysed phosphopeptone (A) freed from H_2SO_4, phosphoric acid, NH_3 and Ba was acidified with H_2SO_4 and continuously extracted with ether for 48–72 hr. The H_2SO_4 was removed quantitatively and the solution after concentration was extracted with butyl alcohol 6–8 times. The butyl alcohol extracts (B) were concentrated *in vacuo* and the solids which crystallized out (B i) were converted into the Cu salts. The Cu salts were separated into two fractions, B i*a* soluble in methanol and B i*b* insoluble in methanol. *iso*Leucine was isolated from the former and serine from the latter. The butyl alcoholic mother liquor (B ii) contained little nitrogenous material.

The aqueous solution (C), after extraction with butyl alcohol, was saturated with HCl gas and glutamic acid hydrochloride (C i) was obtained. The filtrate from this (C ii), after removal of chloride, was concentrated and treated with alcohol to turbidity and cooled, when crystalline serine (C ii*a*) separated out. An uncrystallizable mother liquor (C ii*b*) containing 15·95 % of the total N was left.

Hydrolysis-ether extraction. 60 g. of the Ba salt were hydrolysed with H_2SO_4 and Ba, PO_4, SO_4 and NH_3 were removed as before. The $BaSO_4$ and Ba phosphate contained respectively 4·17 and 1·2 % of the total N. The solution was concentrated *in vacuo* to about 50 ml., acidified with about 5 ml. of 10*N* H_2SO_4 and extracted with ether in a continuous extractor for 72 hr., after which the dicarboxylic acid-N determined on an aliquot after quantitative removal of H_2SO_4 was found to be 30·0 % of the total N. Further extraction did not reduce the titratable acidity. The ether extract was evaporated in the presence of about 30 ml. of water and the aqueous solution was made up to a volume of 50 ml. It contained only 2·1 % of the total N but required 258 ml. of *N*/10 NaOH for neutralization, corresponding to a dicarboxylic acid-N value of 13·6 %.

Butyl alcohol extraction—isoleucine as Cu salt. The ether-extracted hydrolysate was quantitatively freed from H_2SO_4, concentrated to 100 ml. and extracted six times by shaking for 6 hr. each with 300 ml. portions of butyl alcohol. The butyl alcohol extracts were evaporated in three lots and the crystalline material which separated contained respectively 0·5723 g., 0·1168 g. and 0·0549 g. of N (5·45, 1·12 and 0·5 g. of solid respectively). The N contents were 10·51, 10·62 and 10·98 %. They showed no titratable acidity but contained respectively 9·61, 16·43 and 16·9 % of serine-N. The solids were combined and converted into the Cu salts as already described and separated into two fractions, one of Cu salts soluble in methanol (7·6 g.) and the other insoluble (0·98 g.). The methanol-soluble substance on recrystallization from water readily yielded crystalline Cu *iso*leucine: N 8·45 %; Cu 19·58 % (calc. for Cu *iso*leucine, N 8·47 and Cu 19·51 %). After removal of Cu by H_2S the amino-acid was obtained in pure condition with N 10·67 % and $[\alpha]_D^{32^\circ} = +25{\cdot}76^\circ$ in 20 % HCl for a 3·88 % solution.

The methanol-insoluble, water-soluble Cu salt after decomposition with H_2S was found to consist chiefly of serine as determined by the oxidation method; it was therefore added to the next fraction (C).

The material extracted by butyl alcohol was almost completely crystallizable, the final mother liquor (Bii) containing only 3·4 % of the total N.

Glutamic acid hydrochloride. The aqueous solution, (C) after extraction with butyl alcohol (which contained 51·82 % dicarboxylic acid-N and 41·62 % serine-N), was concentrated to a small volume, cooled in ice and saturated with dry HCl gas. After 24 hr. in the refrigerator the separated glutamic acid hydrochloride (Ci) was filtered; from the concentrated filtrate a second crop was similarly obtained. The two fractions (9·18 g.) together contained 27·28 % of the total N. The N content of the mixed solid was 7·7 %. A portion of the substance was recrystallized by dissolving in water and saturating in the cold with dry HCl gas when pure crystals were obtained: N 7·63 %, M.P. 213°. 0·1100 g. of the substance gave 0·0867 g. of AgCl. The molecular weight of the substance was 181·8. The molecular weight of glutamic acid hydrochloride is 183·5.

Serine. The filtrate from the glutamic acid hydrochloride (Cii) was freed from HCl by repeated evaporation to dryness *in vacuo* with water and finally by means of Ag_2SO_4. After removal of H_2SO_4 quantitatively by means of $Ba(OH)_2$, serine was crystallized out by the addition of alcohol as described before. The mixed crops (Cii*a*) weighed 2·2 g. and contained 12·98 % N. A sample recrystallized from aqueous alcohol contained 13·23 % N and melted at 228°. 40 mg. of the substance gave 120·7 mg. of 2:4-dinitrophenylosazone, while the same

Table 6. *Analyses of fractions in scheme II*

	Fractions	N of fractions: In g.	N of fractions: As % of N of fraction	N of fractions: As % of total N of hydrolysate
A.	Hydrolysate	2·5460	—	100·00
	Dicarb.-N	0·7610	—	29·97
	Serine-N	0·7358	—	28·90
	Ammonia-N	0·2774	—	10·90
Bi.	iso*Leucine* (5·84 g.)	0·6229	—	24·46
Bii.	Butyl alcohol mother liquor	0·0860	—	3·38
C.	Aqueous fraction after butyl alcohol extraction	1·5561	—	61·12
	Dicarb.-N	—	51·82	—
	Serine-N	—	41·62	—
Ci.	*Glutamic acid hydrochloride* (9·18 g.)	0·6942	—	27·28
Cii.	Filtrate from above	0·8467	—	33·25
	Dicarb.-N	—	10·45	—
	Serine-N	—	75·04	—
Cii*a*.	*Serine* (2·2 g.)	0·2796	—	10·98
Cii*b*.	Mother liquor from above	0·5671	—	22·27
	Dicarb.-N	—	15·48	—
	Serine-N	—	62·74	—
	Amino-acids from above by scheme I method			
	Glutamic acid (0·2 g.)	0·0191	—	0·75
	iso*Leucine* (0·69 g.)	0·0878	—	3·45
	Serine (0·4 g.)	0·0540	—	2·12
	Final uncrystallizable syrup	0·4062	—	15·95
	Dicarb.-N	—	16·95	—
	Serine-N	—	74·30	—

amount of serine gave 118·4 mg. of the osazone. The three osazones were prepared and the M.P. were identical with those of the osazones obtained from B ii *a* and C ii *a* in scheme I.

From the mother liquor (C ii *b*), by adopting the method of separation described in scheme I, 0·2 g. of glutamic acid, 0·69 g. of *iso*leucine and 0·4 g. of serine were obtained. The final mother liquor, which contained 15·95 % of the total N of the hydrolysate, was uncrystallizable. The results are summarized in Table 6.

Effect of ether extraction on the 'dicarboxylic acid-N' of hydrolysates

The hydrolysate obtained from 20 g. of the Ba salt, after determination of NH_3, was carefully freed from NH_3 and all reagents quantitatively made up to 100 ml. and portions were used for the following experiments. (i) In 2 ml. aliquots the apparent 'dicarboxylic acid-N' was determined by titration to *p*H 7 against *N*/10 NaOH. (ii) 20 ml. aliquots were acidified with 2 ml. of 5*N* H_2SO_4, continuously extracted with ether and the residual acidity determined by titration after removal of H_2SO_4 quantitatively. The ether extract was evaporated, the residue taken up in water and titrated to *p*H 7. (iii) In a 50 ml. aliquot glutamic acid was isolated as the hydrochloride. The results obtained, expressed as *N*/10 BaOH for the whole of the solution, were:

	ml. of *N*/10 NaOH
Total acidity	418·0
Acidity after extraction with ether	286·4
Acidity in the ether-extractable material	122·0
Glutamic acid as hydrochloride	268·7
Ammonia	128·3

The approximate equivalence of the acidity after ether extraction to the glutamic acid isolated and that of the ether-extractable acidity to the ammonia formed during the hydrolysis are patent.

Nature of the ether-extractable material

The fatty acids in the ether extracts did not consist entirely or even mainly of the acids observed by previous workers among the decomposition products of serine. Abderhalden & Broich [1933], who studied the decomposition of serine by H_2SO_4, obtained pyruvic acid, lactic acid, oxalic acid, glycine, alanine and NH_3, while Daft & Coghill [1931] obtained all the above-mentioned products except pyruvic acid during the decomposition of serine by alkali.

The aqueous solution from the ether extract, obtained as above, contained pyruvic acid, but estimation as the 2:4-dinitrophenylhydrazone [Simon & Neuberg, 1931] showed that only 15 % of the total acidity of the extract was due to this acid. Lactic acid estimated by the method of Friedemann *et al.* [1927], as modified by Leib & Zacherl [1932], was found to be present to the extent of 7 %. Thus the major portion of the mixture remained unaccounted for. The extract did not give any colour reactions for the common fatty acids. Ammoniacal $AgNO_3$ was not reduced. A brucine salt was prepared but could not be obtained crystalline. A crystalline quinine salt identical in properties with those of the quinine salt of *l*-glyceric acid described by Nef *et al.* [1917] was, however, obtained in good yield by the following procedure: the aqueous solution of the ether-extractable material was treated with a slight excess of 2:4-dinitrophenylhydrazine dissolved in an equal volume of 4*N* H_2SO_4 to remove pyruvic acid. The precipitated hydrazone was filtered off, and the filtrate was made alkaline with

an excess of $Ba(OH)_2$ and filtered. The solution was extracted repeatedly with ethyl acetate to remove excess of the phenylhydrazine, boiled for a few minutes to remove ethyl acetate and freed from Ba quantitatively by H_2SO_4. The solution was concentrated *in vacuo* and treated on the water bath with an alcoholic solution of quinine drop by drop till the reaction was alkaline. After keeping on the water bath for about 30 min. the solution was cooled, and the excess of quinine was removed by extraction with ether. On concentration *in vacuo* a crystalline mass of the quinine salt was obtained, which was recrystallized from water.

The M.P. of the salt was 164° (165° given by Nef *et al.* [1917]), which was not affected by admixture with the quinine salt of authentic glyceric acid (prepared from serine by deamination with nitrous acid). The weight of the quinine salt obtained from the ether extract (0·35 g. from an aqueous solution requiring 12·6 ml. of *N*/10 NaOH for neutralization) suggests that the main fatty acid produced is glyceric acid. For a more exact determination of glyceric acid the method suggested by Rapoport [1935] was tried. According to this author serine is deaminated with nitrous acid to glyceric acid, which is then estimated from the blue colour formed on treatment with naphthoresorcinol dissolved in conc. H_2SO_4. In our experiments no blue colour was obtained with naphthoresorcinol either with the material from the ether extract or with serine itself treated according to the method described. Serine prepared in this laboratory from silk and a sample obtained from Hoffmann-la Roche were tried; the naphthoresorcinol was also obtained from the same firm.

As Friedemann *et al.* [1927] have shown that glyceric acid can give rise to lactic acid in their method, the lactic acid value mentioned previously does not provide conclusive evidence of the presence of this acid in the ether-extractable material.

The ether extract contained 2·1 % of the total N of the hydrolysate which, even if it was wholly a dicarboxylic acid, would not account for more than about 15 % of the total acidity. Oxidation of the ether extract with chloramine-T and treatment of the resulting solution with 2:4-dinitrophenylhydrazine did not give an osazone.

Table 7. *Time-course of NH_3 liberation during hydrolysis*

	NH_3 as % of total N	
Time in hr.	With 25 % H_2SO_4	With *N* HCl
1	—	0·34
2	—	1·05
4	—	1·60
6	7·88	1·89
8	8·73	2·70
12	9·80	3·97
18	10·92	5·91
24	15·20	6·86

Determination of serine by oxidation with chloramine-T

The method used was a modification of those of Dakin [1918] and Gulland & Morris [1934] for the estimation of 'hydroxyglutamic acid'.

5 ml. of a solution containing 40 mg. of serine (1 mol.) were treated with 0·11 g. of chloramine-T (1 mol.) and the mixture kept on a water bath (60–70°) for an hour. It was then cooled in ice, filtered and the precipitate washed with ice-cold water. The filtrate and the washings were diluted to about 40 ml. and 0·22 g. (3 mol.) of 2:4-dinitrophenylhydrazine dissolved in 40 ml. of warm 4 *N*

HCl was added. The mixture was kept in a boiling water bath for about an hour with occasional stirring, after which the precipitated osazone was filtered hot through a weighed sintered glass crucible and washed thoroughly, first with 4*N* HCl and then with boiling water till the washings were colourless. The precipitate, dried to constant weight, weighed 0·1207 g., which was 75·5 % of the theoretical yield. This proportion was found to be constant in a large number of experiments, including those with mixtures of glutamic acid, *iso*leucine and serine, showing only a variation from about 74 to 76 %, so that using this factor the method can be used for the determination of serine in solutions free from other hydroxy-amino-acids with an experimental error of about 1·5 %.

In the case of mixtures of amino-acids the solution was first neutralized to litmus with alkali before the addition of chloramine-T.

Precipitability of serine by $Ca(OH)_2$ or $Ba(OH)_2$ and alcohol

0·20 g. of serine (containing 26·66 mg. N) was dissolved in 6 ml. of saturated $Ba(OH)_2$ solution in a centrifuge bottle and 60 ml. of alcohol were added slowly from a dropping funnel with vigorous shaking. The mixture was centrifuged for 5 min., the sticky precipitate washed twice with 6 ml. portions of alcohol, dissolved in 6 ml. of water and precipitated once again by gradual addition of 60 ml. of alcohol as before. On dissolving the precipitate and making up to volume (50 ml.) it was found to contain 63·78 % (17·03 mg.) of the N. The filtrate from the Ba-alcohol precipitate was freed from alcohol by evaporation *in vacuo* and contained 37·24 % (9·93 mg.) of the N.

Repetition of the experiment with $Ca(OH)_2$ and alcohol gave similar results.

Summary

By digestion of 'paranuclein' from casein with trypsin an enzyme-resistant phosphopeptone of constant composition has been isolated in the form of its Ba salt.

The phosphopeptone has been shown to contain 10 amino-acid units, viz. 3 mol. of glutamic acid, 3 mol. of *iso*leucine and 4 mol. of serine. The absence of other hydroxy- or dicarboxylic-amino-acids has been demonstrated by indirect methods.

A method is described for the approximate estimation of serine in the absence of other hydroxyamino-acids.

It is shown that the presence of serine in high concentration interferes with the estimation of the dicarboxylic acids both by titration and by precipitation according to Foreman, in the former by the formation of secondary acidic decomposition products and in the latter on account of the partial precipitation of serine under the same conditions as the dicarboxylic acids.

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14. THE ACTION OF SULPHITES ON THE CYSTINE DISULPHIDE LINKAGES IN WOOL

2. THE INFLUENCE OF TEMPERATURE, TIME AND CONCENTRATION ON THE REACTION

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A study of the interaction of wool at room temperature with buffered sulphite solutions of different *p*H [Elsworth & Phillips, 1938, 1] showed that only a fraction of the total disulphide-S in the wool reacted at *p*H 5 according to equation (1) and much smaller amounts in solutions of lower or higher *p*H.

$$R_1.CH_2.S.S.CH_2.R_2 + M_2SO_3 \rightarrow R_1.CH_2.S.M = R_2.CH_2.S.SO_2.OM \quad \ldots\ldots(1)$$

(R_1 and R_2, adjacent polypeptide chains).

The present investigation is concerned with the influence of other factors on this reaction, with the explanation of why it is apparently restricted to only a portion of the total disulphide-S of the wool, and also with the determination of the maximum proportion of the disulphide-S that will react in this manner.

Experimental

The influence of time on the reaction. Pieces of loosely woven, thin woollen fabric, prepared from 64–70's wool, were immersed for the times given in Table 1 in thirty times their weight of buffered $NaHSO_3$ solutions at room temperature containing respectively 1·0 and 2·5 % SO_2; the *p*H was 4·5. The wool fabric was similar to that used in the previous investigation and the buffered $NaHSO_3$ solutions were also prepared in a similar manner. To avoid oxidation, the reactions were carried out in closed flasks. The bisulphited wools were rinsed in water to remove buffer salts and the bulk of the free bisulphite, dried at room temperature and then allowed to reach equilibrium with a controlled atmosphere of 21° and 70 % R.H. Reaction (1) is reversible and bisulphited wool loses $NaHSO_3$ slowly when rinsed in water. For this reason, no attempt was made to wash bisulphited wools until they were free from $NaHSO_3$. The presence of free $NaHSO_3$ does not interfere with the methods of analysis employed [Elsworth & Phillips, 1938, 1, 2]. The conditioned wools were cut up into small pieces, their moisture contents determined by Barritt & King's method [1926] and their thiol-S contents, after hydrolysis for 4 hr. in 5 *N* HCl (20 ml. per g.), by Shinohara's [1935, 1] modification of Folin & Marenzi's method [1929]. Two thiol groups arise in the hydrolysate of the wool for each disulphide group which reacts with the bisulphite, since the *S*-cysteinesulphonate group (equation (1)) is converted into a thiol group when the bisulphited wool is hydrolysed [Elsworth & Phillips, 1938, 1].

$$R_2.CH_2.S.SO_2.OM + HCl \rightarrow R_2.CH_2.SH + MCl + H_2SO_4. \quad \ldots\ldots(2)$$

Table 1. *The influence of time of treatment on the reaction between wool and solutions of $NaHSO_3$ at p*H *4·5 containing respectively 1·0 and 2·5 % SO_2*

Time of treatment hr.	Thiol-S in hydrolysate, % of dry wool: (*a*) From solution containing 1 % SO_2	(*b*) From solution containing 2·5 % SO_2
0·25	0·40	—
0·50	0·44	0·52
1	0·50	0·58
2	0·53	0·64
4	0·55	0·66
7	0·61	0·68
24	0·72	0·73
48	0·69	0·85
96	—	0·83
120	0·64	—
168	—	0·82
288	0·84	—
336	—	0·94

The results (Table 1) show that the rate of reaction is high in the early stages, but then gradually decreases. In both solutions, the reaction proceeds very slowly after 0·7 % of disulphide-S (calculated on the dry wool) has reacted.

The influence of temperature on the reaction. Pieces of the woollen fabric, after previous wetting-out, were heated for 30 min. with forty times their weight of buffered $NaHSO_3$ solutions of *p*H 4·9 containing 1 % of SO_2. They were then rinsed and dried, as described above, before analysis. Besides the thiol-S, the total SO_2 contents of the wools were determined by distillation with dilute H_3PO_4 (1 ml. H_3PO_4, sp. gr. 1·75 in 500 ml.; initial *p*H 2·1) [Elsworth & Phillips, 1938, 2]. The rinsed wools could not be considered to be free from $NaHSO_3$, so that a small amount of the total SO_2 returned by this method was derived from this source. The bulk of the SO_2 arose from the decomposition of *S*-cysteine-sulphonate groups according to equation (3).

$$R_2.CH_2.S.SO_2.ONa \xrightarrow{H_3PO_4} R_2.CH_2.SOH + NaHSO_3. \qquad \text{.....(3)}$$

Table 2. *The influence of temperature on the reaction between wool and a solution of $NaHSO_3$ at p*H *4·9 containing 1 % SO_2*

Temp. ° C.	Total SO_2, % of dry wool	Thiol-S in hydrolysate, as % of dry wool
20	0·72	0·45
30	0·83	0·62
40	0·75	0·65
50	0·83	0·69
60	0·97	0·72
70	0·98	0·74
80	1·01	0·76
90	0·91	0·76
100	0·75	0·66

From Table 2 it will be seen that increase in temperature up to 60° assists the reaction. At higher temperatures the wool was weakened, and at 100° it is probable that other chemical changes occurred in the wool besides the bisulphite reaction and caused the observed decrease in the thiol-S in the hydrolysate. Theoretically, since the weight of 2S atoms is the same as that of the SO_2 molecule, the weights of SO_2 obtained from these bisulphited wools should have

equalled the weights of thiol-S found in the hydrolysates. In every wool, the total SO_2 is greater than the thiol-S because of the incomplete removal of the uncombined $NaHSO_3$ before analysis.

The influence of the concentration of $NaHSO_3$. Pieces of the woollen fabric (10 g.) were immersed for 24 hr. at room temperature in 300 ml. of buffered $NaHSO_3$ solutions (*p*H 5·3–5·7) containing 0·1–5·0 % SO_2. The subsequent treatment of the wools before the determination of the thiol-S contents of their hydrolysates was as previously described. In Fig. 1 the thiol-S contents of the

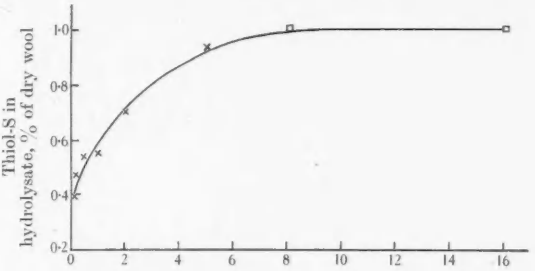

Fig. 1. SO_2, % in solution, *p*H 5·3–5·7. ×—× Commercial fabric. □—□ Australian 64's.

hydrolysates of the bisulphited wools are plotted against the concentrations of the solutions in which the wools were treated. It will be seen that the extent of the reaction increases at first, but reaches a maximum when the solution contains 8 % SO_2 (13 % $NaHSO_3$).

These observations were extended using a virgin wool which contained very nearly the same percentage of disulphide-S as the wool of the commercial fabric. This virgin wool was prepared from a 64's Australian merino fleece by extracting a picked portion three times with benzene at 40° and then removing dirt, vegetable matter and discoloured fibres by hand. The remaining wool, after it had been extracted three times with benzene and twelve times with water, was finally washed in an 0·05 % solution of saponin followed by twelve rinses in water. After drying at room temperature and allowing to come to equilibrium with a controlled atmosphere of 21° and 70 % R.H., the clean wool contained 3·42 % total S and 3·09 % disulphide-S (calculated on its anhydrous weight). Samples of this wool were immersed for 24 hr. in fifty times their weight of solutions of $NaHSO_3$ of *p*H 5·7 containing respectively 8 and 16 % SO_2. The thiol-S values found in the hydrolysates of these bisulphited wools are plotted in Fig. 1, and it will be seen that they fall on the curve drawn through the points given by the wool of the commercial fabric. This wool contained 3·60 % S and 2·99 % disulphide-S. The curve in Fig. 1 possibly gives an exaggerated idea of the regularity of the reaction. A wider variety of different wools needs to be examined before the precise fraction of the disulphide-S that reacts with $NaHSO_3$ can be stated definitely.

After both these wools had been bisulphited, their original disulphide-S could be almost entirely accounted for either as thiol-S or as disulphide-S: hence during bisulphiting at room temperatures no conversion of disulphide-S into non-disulphide-S[1] took place. For example, after exposure for 24 hr. to the

[1] The term non-disulphide-S is used throughout this paper to describe S not returned either as thiol-S or as disulphide-S by Shinohara's method or as methionine-S by Baernstein's method.

$NaHSO_3$ solution containing 8 % SO_2, the virgin wool when hydrolysed gave 1·01 % thiol-S and 1·93 % disulphide-S, thus accounting for 2·94 % S or nearly all the total of 3·09 % S originally present as disulphide. This conclusion is of interest in the light of analytical results obtained when more drastic conditions for bisulphiting wool were used. Samples of the virgin wool were immersed in $NaHSO_3$ solutions at *p*H 5·7 containing 6·2 % SO_2 which were maintained at 40°. After $5\frac{3}{4}$ and also after 19 days' exposure to this solution, the wools gave 1·18 and 1·12 % thiol-S respectively. These percentages are somewhat higher than those produced by the treatment of wool with $NaHSO_3$ at room temperature. It is doubtful, however, whether this increase in thiol-S is due to an increase in the reaction between the wool and $NaHSO_3$ according to equation (1). At the higher temperature, other reactions occur which may increase the amount of thiol-S produced.

The occurrence of such reactions is shown by the conversion of disulphide-S into non-disulphide-S which took place. Thus the original wool contained 3·09 % disulphide-S, but after $5\frac{3}{4}$ days' treatment in $NaHSO_3$ at 40° only 2·76 %, and after 19 days only 2·49% of S in the wool could be accounted for either as disulphide- or thiol-S after hydrolysis.

The treatment of wool with concentrated solutions of $NaHSO_3$ (containing 8 % or more of SO_2) at room temperatures thus appears to lead to a straightforward reaction in which about one-third of the disulphide-S is converted into thiol and *S*-cysteinesulphonate groups. If the temperature of the $NaHSO_3$ solution is raised, even to 40°, other changes take place slowly and disulphide-S is converted into S which is neither thiol-S nor S present in *S*-cysteinesulphonate groups.

The reaction between $NaHSO_3$ and the cortical cells of wool. Although wool is attacked very slowly by a solution of papain, it has been found [Phillips, Middlebrook & W.I.R.A., 1939] that when $NaHSO_3$ is added to the papain solution, the wool is rapidly reduced to a mass of cortical cells, the intercellular protein and scales being digested. Cortical cells produced by this method were used to obtain evidence that they react with $NaHSO_3$. A 64's merino wool, which had been scoured commercially, was extracted with alcohol and then rinsed repeatedly in water. A sample of the wool was immersed for 30 min. in forty times its weight of solution of $NaHSO_3$ at *p*H 5·7 containing 2·0 % SO_2. The wool and solution were maintained at 65°. The bisulphited wool was rinsed with water and then analysed for thiol-S, free and combined SO_2 and for sulphate-S. A further sample of the wool (40 g.) was digested at 65° in 1 l. of 0·05 % papain solution containing 1 % $NaHSO_3$. After 17 hr., the digest was centrifuged and the insoluble material digested for a further 17 hr. with a fresh enzyme solution. The cortical cells that remained were centrifuged repeatedly with water until the supernatant liquor no longer gave a turbidity on the addition of H_2O_2 and $BaCl_2$. After drying *in vacuo* over P_2O_5 to constant weight, they were analysed for total S and thiol-S. They did not contain either free or combined SO_2, indicating that the bisulphite which they acquired in the enzyme solution had been removed completely. A sample of the cortical cells was then bisulphited under exactly the same conditions as the wool. The bisulphited cells were removed by centrifuging the solution and were washed with water. After drying *in vacuo* over P_2O_5, they were analysed again, the results of this analysis and those of the previous analyses being given in Table 3 as a percentage of the dry wool and cells. The total and 'free' SO_2 were determined by Elsworth & Phillips' methods [1938, 2].

Table 3. *Analysis of wool and wool cortical cells before and after treatment with $NaHSO_3$*

	Total S %	Thiol-S in hydrolysate %	Free SO_2 %	Combined SO_2 %	Sulphate-S %
Wool	3·64	0·10	—	—	0·08
Wool after bisulphiting	4·21	0·76	0·0	0·74	0·28
Cortical cells	4·12	0·17	0·0	0·0	—
Cortical cells after bisulphiting	5·09	0·75	0·23	0·76	0·66

The analyses of thiol-S, after hydrolysis, and the combined SO_2, show that very nearly the same percentages of the disulphide-S of the wool fibre and of the cortical cells react with $NaHSO_3$. This agreement may be accidental, since changes in the disulphide-S of the wool and cells, leading to its conversion into unreactive non-disulphide-S, may have occurred at unequal rates when the wool and cells were warmed with $NaHSO_3$ at 65° for 30 min. In addition, during their isolation the cortical cells were exposed for two periods of 17 hr. to 1 % $NaHSO_3$ at 65°.

The data of Table 3 provide some evidence as to the S content of the cortical cells and how this compares with that of the whole wool fibre. The S content of the cells can be calculated from the analyses after sulphiting, since these include a determination of sulphate-S by Mease's [1934] method. Allowing for sulphate-S, and S present as free and combined SO_2, the S content of the cells is 3·94 % calculated on their anhydrous weight. This exceeds the organic S, namely 3·56 %, of the wool from which they were isolated. Other evidence is available showing that the S content of the cellular components of the wool fibre exceeds that of the intercellular material. Stakheyewa-Kaverznewa & Cavrilov [1937] obtained an extract by the pancreatin-enterokinase digestion of wool which contained less S than the residual cells. They concluded that wool contained some 10 % of an intercellular phase of low S content. Speakman & McMahon [1938] digested the root portions of non-medullated Australian wool fibres with trypsin (thus avoiding the presence of light-damaged fibres with might be preferentially attacked by the enzyme) and found that the residual wool had a higher S content than the original wool. Their figures agreed with the presence of some 9 % of S-free protein in wool.

The reaction between alkali-degraded wool and $NaHSO_3$. On the hypothesis that $NaHSO_3$ reacts with a labile fraction of the disulphide-S of wool, it was thought possible that such S could be removed preferentially by alkali and the wool would not then react to the same degree, if at all, with $NaHSO_3$. The wool for these experiments was prepared by immersing pieces of the wool fabric for 30 min. in boiling buffer solutions of *p*H 8–10. The buffer solutions were borax-boric acid buffers and were 0·2 *M* in borate ion, except those of *p*H 9·6 and 10·0, which were 0·15 and 0·12 *M* respectively. The total S contents of the untreated and treated wools, after these had been rinsed thoroughly with water, were determined by the Denis-Benedict method as applied to wool by Barritt [1934]. The disulphide-S remaining intact in the wools was determined by Shinohara's method [1935, 2]. The pieces of fabric (about 10 g.) were then immersed for 20 hr. in 2800 ml. of a solution of $NaHSO_3$ at *p*H 5·0 containing 1·5 % SO_2. After passing through a wringer, the wools were immersed in water for 1 hr., allowed to dry in air and then to come to equilibrium with a controlled atmosphere of 21° and 70 % R.H. They were then analysed for thiol-S, and free and total SO_2, from which the combined SO_2 content was calculated.

Table 4. *Analyses of alkali-treated wools before and after reaction with $NaHSO_3$*

		After alkaline treatment followed by reaction with $NaHSO_3$			
(i) *p*H of buffer with which wool was boiled	(ii) Total-S, % after treatment	(iii) Total SO_2 %	(iv) Free SO_2 %	(v) Combined SO_2 %	(vi) Thiol-S in hydrolysate %
(Untreated)	3·60	0·79	0·23	0·56	0·61
10·0	2·52	0·45	0·21	0·24	0·22
9·6	2·90	0·47	0·21	0·26	0·28
9·2	3·05	0·53	0·21	0·32	0·37
8·9	3·29	0·55	0·09	0·46	0·45
8·5	3·56	0·64	0·17	0·46	0·47
8·1	3·51	0·69	0·16	0·53	0·53

An important deduction from these results (Table 4) is that the amount of reaction that occurs is proportional to the disulphide-S contents of the wools as recorded in Table 5. Thus the thiol-S values in the hydrolysates of the wools (Table 4, col. (vi)) are approximately one-fifth of the disulphide-S contents of the wools (Table 5, col. (ii)), suggesting that no matter how small the disulphide-S becomes, one-fifth will react with $NaHSO_3$ under the conditions of these experiments. The residual disulphide-S of alkali-treated wools thus appears to react as freely with $NaHSO_3$ as the disulphide-S of untreated wool.

Another significant feature of these results is that for each thiol group in the alkali-treated and bisulphited wools there is one *S*-cysteinesulphonate group. This is indicated by the very good agreement between the figures for the combined SO_2 and the thiol-S in the hydrolysates (Table 4, cols. (v) and (vi)). When the extent and possible nature of the chemical alteration produced in the wool by alkalis is taken into consideration, this agreement is remarkable. For example, most of the chemical changes which it has been suggested that wool undergoes when treated with alkalis are assumed to give rise to free thiol groups. Speakman [1933] has suggested that mild treatment with alkali may cause hydrolysis of the disulphide linkage, followed, under certain conditions, by condensation between the sulphenic acid groups and adjacent amino-groups.

$$.CH_2.S.S.CH_2. \rightarrow .CH_2.SH + .CH_2.SOH, \quad \text{.....(4)}$$

$$.CH_2.SOH + H_2N. \rightarrow .CH_2.SNH. + H_2O, \quad \text{.....(5)}$$

$$.CH_2.SOH \rightarrow .CHO + H_2S. \quad \text{.....(6)}$$

These changes ((4) and (5)) occur without loss of S. When S is lost (as in (6)) Schöberl [1933] has suggested that the sulphenic acid groups decompose giving aldehyde groups and H_2S. Race *et al.* [1938] have obtained evidence that a decomposition similar to that postulated by Schöberl occurs in the tips of wool fibres when they are exposed to light and moisture during growth. They found that such light-damaged wool contained aldehyde groups, but less S than the corresponding root wool. Speakman & McMahon [1938] titrated wool from the tips and from the root ends of a fleece and from the differences between the titration curves concluded that the tips of the fibres contained appreciable quantities of thiol groups, whereas the root ends contained only traces. The evidence for the presence of thiol groups in wool which has undergone chemical change in aqueous solutions is not so strong. Crowder & Harris [1936] caused wool to lose half its S, in accordance with the Schöberl reaction, by exposing it to a current of aqueous NaOH. They were unable to find any cysteine in the hydrolysate of the wool and assumed that this was due to the formation of thioacetals during the hydrolysis.

Assuming that disulphide-S is destroyed by the reactions suggested by Speakman and Schöberl, an estimate of the amounts of thiol-S which were formed in the alkali-treated wools can be made from the analyses recorded in Tables 4 and 5. In these analyses, the difference between the values of the disulphide-S and total S minus the value of the methionine-S (found to be 0·10 % using Baernstein's method [1932] for the determination of methionine) gives the amount of S present in the wools as non-disulphide-S. In the untreated wool, the non-disulphide-S amounts to 0·51 % on the weight of the anhydrous wool, and is considered to have arisen without loss of S. This may not be true, since the wool may have lost S by exposure to air, moisture and light during growth, but the amount is unlikely to be large, since the total S of the wool is 3·60 %, a reasonably high value. The total non-disulphide-S in the alkali-treated wools is subdivided by assuming that for each atom of S lost by the wool, one atom of non-disulphide-S is produced. The non-disulphide-S produced with loss of S is then subtracted and figures for the non-disulphide-S produced without loss of S are obtained.

Table 5. *The modification of the disulphide-S of wool when it is treated with boiling buffer solutions*

*p*H of buffer with which wool was boiled	Disulphide-S, % after treatment	Non-disulphide-S, % produced without loss of S	Non-disulphide-S % produced with loss of S
—	2·99	0·51	—
8·1	2·59	0·73	0·09
8·5	2·36	1·06	0·04
8·9	1·87	1·01	0·31
9·2	1·57	0·83	0·55
9·6	1·31	0·79	0·70
10·0	1·04	0·30	1·08

It will be seen (Table 5) that when wool is treated with boiling mildly alkaline solutions relatively large amounts of non-disulphide-S are produced without loss of S: in the more strongly alkaline solutions, non-disulphide-S is produced with loss of S. The significance of these changes will be more fully discussed in a subsequent paper. Here we need only point out that if the non-disulphide-S was produced according to the Speakman and Schöberl reactions, part of it should have been found as thiol-S. For example, the hydrolysate of the wool boiled with the buffer of *p*H 9·2 should have contained 0·42 % thiol-S arising by the Speakman reaction and 0·55 % thiol-S by the Schöberl reaction, a total of 0·97 %, calculated on the weight of wool hydrolysed. Experimentally none was found. On the other hand, when this alkali-treated wool was bisulphited, and determinations of the combined SO_2 indicated that theoretically the hydrolysed wool should contain 0·32 % thiol-S, 0·37 % was actually found.

In some instances, it is not possible to attribute the failure to find the thiol-S to the presence of aldehydes in the hydrolysate of the wool. For example, the non-disulphide-S in the wool increased from 0·51 to 1·06 % when it was boiled in buffer of *p*H 8·5, but the wool lost only 0·04 % S. If the additional non-disulphide-S arose mainly by the Speakman reaction, 0·25 % thiol-S was produced without the simultaneous production of aldehyde groups. In spite of this, no increase in the thiol-S of the hydrolysed wool was observed. It appears probable therefore that if disulphide cross-linkages are destroyed according to the Speakman and Schöberl reactions, the thiol-S produced must become linked to other atoms in the keratin molecule.

Discussion

The present investigation has shown that whilst the reaction between wool and $NaHSO_3$ proceeds quickly at first, there is a point at which it is difficult to cause further reaction to take place. This is brought out most clearly in Fig. 1, which shows the effect of increasing the concentration of the $NaHSO_3$. In solutions containing up to 8 % SO_2 at room temperatures about one-third of the disulphide-S of the wool reacts and a twofold increase in concentration produces no further reaction. At higher temperatures, although increased amounts of disulphide-S disappear from the wool, there is evidence that the wool has not combined with equivalent amounts of $NaHSO_3$.

It is, however, important to emphasize that all the bisulphited wools mentioned in this paper were analysed after they had been rinsed with water. It is known [Elsworth & Phillips, 1938, 1] that $NaHSO_3$ combined with wool is not readily removed by water, but further work is necessary to prove whether all the $NaHSO_3$ removed when bisulphited wool is rinsed with water is free $NaHSO_3$ and not $NaHSO_3$ less firmly combined with some additional fraction of the disulphide-S.

Nevertheless sufficient evidence has been obtained to suggest that the reactivity of one-third of the disulphide-S of wool towards $NaHSO_3$ differs from that of the remaining two-thirds. This does not necessarily mean that one-third of the disulphide-S of wool is more reactive than the remaining two-thirds. If this were so, we should expect to be able to remove the more reactive fraction by means of alkali, and obtain a wool which would not react with $NaHSO_3$. Experimentally it is found, however, that the residual disulphide-S in alkali-treated wools is as reactive towards $NaHSO_3$ as the disulphide-S of untreated wool.

A more likely hypothesis appears to be that whilst all the disulphide linkages of wool are equally reactive towards $NaHSO_3$, after this reagent has reacted with and broken some of these linkages, stresses on the remaining linkages are dissipated and their reactivity is altered. In agreement with this suggestion, it is known that the physical properties of hair are profoundly changed by boiling it for 30 min. with a 5 % solution of $NaHSO_3$. Speakman [1936] found that such treatment enabled human hair to supercontract 30 % in length when it dried. Assuming that human hair reacts with $NaHSO_3$ in the same manner as wool, it would appear that this supercontraction becomes possible because about one-third of the disulphide linkages in the hair have been broken. These linkages may therefore be responsible for maintaining the keratin in the α-configuration: when they are broken by $NaHSO_3$, the keratin molecule can assume a more folded and shorter configuration [Astbury & Woods, 1933].

Unstretched human hair that has been boiled with a 2 % solution of borax (*p*H 9·2) does not contract in length when boiled with 5 % $NaHSO_3$. If it is first boiled with dilute HCl and then with 5 % $NaHSO_3$, it contracts 15 % in length [Speakman & Stoves, 1937]. These authors assume that for each disulphide linkage destroyed by the borax another linkage is produced. This may either be a —S—NH— linkage arising without loss of S, or, when S is lost, a —CH=NH— linkage arising, as suggested by Phillips [1936], by condensation between the aldehyde group and an adjacent amino-group. Boiling the hair in dilute HCl may break the —CH=N— cross-linkages, so that after additional linkages have been broken by $NaHSO_3$, the total number of linkages broken is then sufficient to enable some supercontraction to take place.

SUMMARY

1. The disulphide-S of wool reacts with $NaHSO_3$ and thiol and *S*-cysteinesulphonate groups are formed which are not readily removed by rinsing the bisulphited wool with water. By increasing the temperature, time of reaction and concentration of $NaHSO_3$ not more than one-third of the total disulphide-S of the wool can be caused to react in this manner.

2. The cortical cells, isolated by digestion of wool with papain in the presence of $NaHSO_3$, contain more organic S than the fibre as a whole. The keratin of the cells reacts with $NaHSO_3$ to give thiol and *S*-cysteinesulphonate groups.

3. The residual disulphide-S in wool that has been boiled with alkaline solutions of *p*H 8 and higher reacts with $NaHSO_3$ in the same proportion as the disulphide-S of untreated wool.

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15. A STUDY OF THE ISOTOPIC CONSTITUTION OF POTASSIUM IN VARIOUS RAT TISSUES

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Preliminary investigations [Brewer, 1936; 1937; Lasnitzki & Brewer, 1938] have disclosed the somewhat surprising fact that the abundance ratio of the isotopes of potassium is not a constant figure for all organic tissues. Since the observed isotope effect is necessarily associated with processes involved in K metabolism, it was considered advisable to make a comprehensive study of the isotope ratio in various animal tissues in hope that it would contribute to our knowledge of the mechanism of K assimilation. Various tissues from the rat, including red blood corpuscles and blood plasma, have been chosen for investigation.

Potassium is known to have three natural isotopes, designated as: $^{39}_{19}$K, $^{40}_{19}$K and $^{41}_{19}$K, the indices '39', '40' and '41' indicating the mass number and the common index '19' the nuclear charge. The 39-isotope represents the bulk of the element, the 41-isotope a relatively small fraction, while the 40-isotope (which is radioactive) exists only in a very minute quantity. The present study is concerned only with the 39- and 41-isotopes.

Methods

Preparation of ashes. Adult or preadult rats, taken from mixed strains, were studied first. The animals were anaesthetized with chloroform, and, after opening the chest, blood was drawn from the heart, usually by puncture of the left ventricle. The blood was cooled on ice, mixed with a small amount of 5% sodium citrate solution to prevent clotting, and then centrifuged at high speed. The citrate plasma was separated, centrifuged again and filtered through an ash-free filter. The red blood corpuscles were freed from residual plasma and white blood cells by washing them several times with 0·9% NaCl. Plasma and blood corpuscles were then dried at about 100°, part in glass and part in silica vessels.

The solid tissues were taken immediately after the recovery of the blood. The soft organs were freed as far as possible from adhering connective tissue, etc., and cut into small pieces; these were rapidly rinsed in distilled water and dried on Petri dishes at the above temperature. The bones were treated similarly. By the cardiac puncture and subsequent section of the venae cavae the blood content of the tissues was lowered appreciably. When no heart puncture was made, the animal was bled in a similar way after being chloroformed.

Some solid tissues were taken also from new-born rats. The animals were killed by decapitation and freed as far as possible from blood. The tissues were treated as above.

After drying, the corresponding material obtained from different animals was mixed and then ashed, wholly or in part. The ashing was carried out in a Pt dish, which had been previously cleansed and ignited to bright redness in order to remove any K possibly absorbed by the metal. The number of animals employed in the preparation of one ash sample varied considerably. Among the adult and preadult animals an equal number of males and females was as a rule utilized.

Mass-spectrographic measurement. The ash prepared in this way was used, without further treatment, for the determination of the isotopic ratio $^{39}_{19}K/^{41}_{19}K$ by means of a mass-spectrograph. The special type of apparatus employed was previously described in detail [Brewer, 1938]. The essential parts are shown schematically in Fig. 1. A Pt disk, 1·5 mm. in diameter, was the source of the K atoms. The disk was spot-welded to a tungsten filament which was heated by a heavy duty storage cell. Before coating, the disk was ignited at white heat until all residual K was removed. A small quantity of ash was then placed on the disk and heated in a vacuum to about 700° for 30 min. During this heating process the Pt absorbed sufficient K to become an excellent emitter of K ions after the ash had been carefully scraped away. The impregnated disk was mounted at a distance of 5–8 mm. directly above the entrance slit to the analysing chamber. This arrangement ensured that all the potassium ions leaving the disk and impinging on the slit had attained the same velocity. Between the disk and the slit a potential drop of 540 v. was maintained, the disk being positive. By surrounding the disk with a washer type shield, maintained positive to the disk by about 60 v., it was possible to bring all the emitted ions to a focus on the slit, and by a special arrangement (not illustrated) the slit could be placed directly in the centre of the path of the focussed ion beam. When the disk was brought to a temperature of about 600°, the positive ion current from the disk to the slit was of the order of 10^{-9} to 10^{-10} amp.

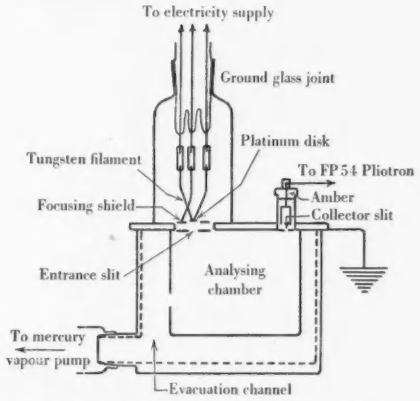

Fig. 1. Diagram of mass-spectrograph.

The ion current entering the analysing chamber was resolved by means of a magnetic field produced by an electromagnet with poles directed against the side walls of the chamber, which were made of Swedish iron. By adjusting the current to the magnet it was possible to bring all the ions of the same mass number to a focus at the collector slit. Since the latter was wide with respect to the entrance

slit, the entire resolved ion beam was caught by the collector. The intensity of each resolved ion current was recorded by means of an FP54 Pliotron. The ratio of the galvanometer deflexions at the 39 and 41 mass positions was equal to the isotopic ratio $^{39}_{19}K/^{41}_{19}K$, since under the given conditions the deflexions were linear over a large scale. From the isotopic ratio the percentage of the heavy isotope $^{41}_{19}K$ was calculated, neglecting the very minute amount of $^{40}_{19}K$.

The most common error occurring in these measurements was associated with the 'background' resulting from the scattering in the resolved ion beams. Since accurate measurements could not be performed in the presence of background, all measurements were excluded in which the galvanometer deflexion at the 40 mass position exceeded 0·1 mm. Another error might have arisen from the platinum disk failing to take up the two isotopes exactly in the same ratio in which they were present in the ash; careful tests showed no evidence for such a possibility. In practice about thirty estimations of the isotopic ratio were always made in succession on each ash sample. While individual estimations showed some variation, the average of any ten estimations usually agreed to within $\pm 0{\cdot}01$, corresponding to $\sim \pm 0{\cdot}005\,\%$ $^{41}_{19}K$.

Results

Ordinary commercial KCl (A.R.) served as a normal standard for the comparison with the K contained in tissues. For the mass-spectrographic analysis the chloride was converted into the perchlorate, which could be used in the same manner as the ash. The isotopic ratio $^{39}_{19}K/^{41}_{19}K$ determined with this preparation was 14·20, the $^{41}_{19}K$ content being 6·579 %. Results obtained with ashes from various tissues are shown in Tables 1–5, each corresponding to one particular group of tissues.

In Table 1 the results from liver, kidney, lung and salivary gland are given. It will be seen that the isotopic ratios are, in the majority of cases, very close to that

Table 1. *Analysis of K in liver, kidney, lung and salivary gland*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Liver	8	210	14·20	6·579
2	,,	4	220	14·27	6·549
3	,,	4	150	14·20	6·579
4	Liver (perfused)	2	235	14·23	6·566
5	Kidney	12	210	14·28	6·545
6	,,	6	215	14·22	6·570
7	,,	4	150	14·20	6·579
8	Lung	12	210	14·24	6·562
9	,,	4	150	14·20	6·579
10	Salivary gl.	16	215	14·31	6·532
11	,, ,,	10	210	14·21	6·575
12	,, ,,	6	145	14·20	6·579

of mineral K. On the other hand, there are three values—one each with liver, kidney and salivary gland—which are very slightly increased, more than can be accounted for by the experimental error. The content of $^{41}_{19}K$ was thus decreased (by 0·5–0·7 %). Ash sample no. 4 is of special interest. The livers used for the preparation of this sample were perfused *in situ* through the portal vein by K-free Ringer's solution with an increased content of Ca. This perfusion

removed almost completely the blood present in the tissue. The effect on the isotopic ratio was, however, negligible or zero.

Table 2 contains the results from skeletal muscle, heart muscle and brain. The values of the isotopic ratio do not show any noticeable deviation from that of mineral K, except one sample from skeletal muscle. Here again a minute increase of the isotopic ratio was observed, and a corresponding decrease of the $^{41}_{19}K$ content (by about 0·4 %).[1]

The tissues dealt with in Table 3, i.e. spleen, lymph glands, testis, and bone including marrow, are distinguished from the above tissues by the greater rate of

Table 2. *Analysis of K in skeletal muscle, heart muscle and brain (cortex and medulla)*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Skeletal muscle	8	210	14·26	6·553
2	,, ,,	4	220	14·20	6·579
3	,, ,,	4	150	14·20	6·579
4	Heart muscle	12	210	14·20	6·579
5	Brain	12	210	14·22	6·570
6	,,	4	155	14·19	6·583

Table 3. *Analysis of K in spleen, lymph glands, testis and bone including marrow*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Spleen	12	210	14·22	6·570
2	Lymph glands	16	225	14·20	6·579
3	Testis	12	250	14·20	6·579
4	Bone including marrow*	8	220	13·95	6·689
5	Do.*	2§	250	13·97	6·680
6	Do.*	2‖	195	13·97	6·680
7	Do.†	4	150	13·79	6·761
8	Do.‡	1§	225	13·88	6·721

* Diaphyses of humerus, femur and tibia of both sides.
† Diaphyses of femurs only.
‡ Diaphyses of left bones only.
§ Males only. ‖ Females only.

proliferation of their cellular elements. It will be seen that with ashes from spleen, lymph glands and testis no noticeable deviation of the isotopic ratio was found. A marked difference, however, was obtained with ashes from bone including marrow. In each of the five samples analysed the isotopic ratio was clearly decreased, and the content of $^{41}_{19}K$ thus increased. The value of this increase showed some variation. In three of the samples it was 1·5–1·7 %, in one sample about 2·2 %, and in one sample about 2·8 %. The average was 1·9 %.

[1] Another analysis not included in the table was carried out with a sample of $KClO_4$, prepared by $HClO_4$ precipitation from an extract of rabbit muscle. No deviation of the isotopic ratio was found. This result agreed with previous findings by Lasnitzki & Oeser [1937] concerning the radioactivity of K in rabbit muscle, a similar preparation (after its conversion into the chloride) having been used.

Further, it was found that ash prepared from a humerus, femur and tibia, after complete removal of the marrow from the cavity, showed an isotopic ratio of 14·03, corresponding to a $^{41}_{19}$K content of 6·653 %. The bones were taken from the right side of the same animal from which the corresponding bones of the left side constituted ash sample no. 8. Thus K contained in the bone substance appears to show a smaller deviation of its isotopic ratio than K derived from both bone substance and marrow. K from marrow alone would show, therefore, rather a greater deviation of its isotopic ratio than that which was actually found.

The conclusion that the K present in bone marrow of the rat has a higher content of $^{41}_{19}$K than ordinary mineral K agrees with similar findings previously obtained with marrow from horse and beef [Brewer, 1937].

The results obtained with red blood corpuscles and blood plasma are shown in Table 4. It is evident that the isotopic ratio of K in the ash of red blood corpuscles was not different from normal. On the other hand, a marked decrease was observed in the ash of blood plasma. The deviation was clearly indicated with each of the five plasma samples, yet with two of them more pronouncedly than with the others. The corresponding increases in the content of $^{41}_{19}$K were 3·0–3·2 and 1·8–2·3 % respectively; the average increase was 2·5 %.

Table 4. *Analysis of K in red blood corpuscles and blood plasma*

Sample no.	Source	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}$K/$^{41}_{19}$K	% of $^{41}_{19}$K
1	Red blood corpuscles	12	220	14·20	6·579
2	,, ,, ,,	6	220	14·19	6·583
3	,, ,, ,,	4	155	14·20	6·579
4	Blood plasma	12	220	13·74	6·784
5	,, ,,	6	220	13·92	6·702
6	,, ,,	6	215	13·72	6·793
7	,, ,,	2	200	13·87	6·725
8	,, ,,	4	155	13·89	6·716

A special test was carried out to determine whether the very high Na content of the plasma ash, due to the mixture of blood and sodium citrate solution, might have interfered with the mass-spectrographic measurement; although there was no theoretical reason for such a possibility. Dry liver and muscle tissue were thoroughly mixed with sodium citrate in a ratio approximating plasma. The ashes of these mixtures and those of the plain dry tissues were compared respectively, but no difference in the isotopic ratio was found. Thus the high preponderance of Na in the plasma ash did not interfere with the measurements. (The same can be said with regard to Ca in bone ash, since the addition of a large amount of calcium phosphate to dry tissue did not affect the isotopic ratio.)

In Table 5 are given the results for liver, kidney and skeletal muscle, taken from new-born animals. While K contained in the ash of liver and muscle tissue showed about the same isotopic ratio as mineral K, that present in kidney tissue showed a minute increase of this value, and thus a slight decrease in the $^{41}_{19}$K content (by about 0·7 %). This deviation is comparable with that obtained with one of the ash samples from adult kidney, but does not agree with the absence of any noticeable deviation in the two other samples. On the whole, no difference seems to exist in the isotopic ratios of K animals in these three tissues in relation to the age of the animal.

Table 5. *Analysis of K in liver, kidney and skeletal muscle of new-born animals*

Sample no.	Tissue	No. of animals used	Average animal wt. in g. (approx.)	Isotopic ratio $^{39}_{19}K/^{41}_{19}K$	% of $^{41}_{19}K$
1	Liver	111	5·5	14·19	6·583
2	Kidney	127	5·5	14·30	6·536
3	Skeletal muscle	7	5·0	14·21	6·575

A representation of the whole of our results is shown in Fig. 2. The height of each column indicates the % of $^{41}_{19}K$ found in K contained in one particular ash sample, while the dotted line indicates the % of $^{41}_{19}K$ in mineral K. The columns are interrupted between 0·2 and 6·4 % $^{41}_{19}K$.

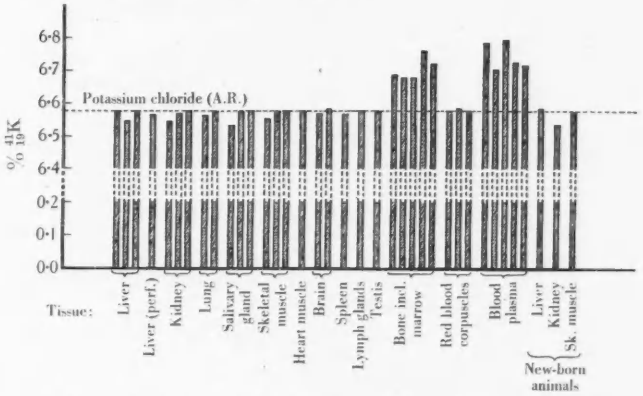

Fig. 2. Content of $^{41}_{19}K$ in potassium present in various rat tissues.

Discussion

Considering the results as a whole, it appears that a constant deviation from the isotopic ratio of mineral K was observed only for K present in bone marrow and blood plasma. In both cases the deviation consisted in a slight but definite increase in the content of the heavy isotope. The K contained in other tissues, i.e. liver, kidney, salivary gland and skeletal muscle, has occasionally shown minute deviations in an opposite direction.

In the living animal a continuous K exchange takes place between the various tissue cells and the blood plasma, the latter being probably in direct communication with the intercellular spaces. The exchange occurs in both directions, and generally at a slow rate. Further, the actual K content of the majority of tissues is very much (on an average perhaps twenty times) higher than that of the blood plasma, and this difference is maintained independently of the K exchange between cell and plasma. Although no definite picture of the mechanism of this exchange is available, it is certain that while K is specifically absorbed by the cell body, it remains in a state of dynamic equilibrium with the K in the surrounding medium. Under these conditions the appearance of an isotope effect will be possible.

A simple kinetic mechanism can be devised to account for the difference in the isotopic ratios of K in tissue (excluding bone marrow) and plasma. This mechanism depends on the difference in velocity of movement of the K atoms in the two media concerned. As far as we are aware, K atoms in the plasma and the medium directly surrounding the cell exist as free ions. The ratio of the numbers of $^{39}_{19}$K and $^{41}_{19}$K atoms striking the outer surface of the cell membrane, and entering thereby the cell, is then given by

$$n_{39}/n_{41} = \frac{C_{39}}{\sqrt{39}} \Big/ \frac{C_{41}}{\sqrt{41}}, \qquad \ldots\ldots(1)$$

where C_{39} and C_{41} denote molar concentrations of $^{39}_{19}$K and $^{41}_{19}$K in the plasma. Since a state of equilibrium exists, K atoms are also moving in the reverse direction. In the cell, in contrast to the plasma, K is associated with heavy molecular groups of mass M (possibly proteins); in consequence the ratio of the numbers of $^{39}_{19}$K and $^{41}_{19}$K atoms striking the inner surface of the cell membrane, and thereby leaving the cell, is expressed by

$$n'_{39}/n'_{41} = \frac{C'_{39}}{\sqrt{(M+39)}} \Big/ \frac{C'_{41}}{\sqrt{(M+41)}}, \qquad \ldots\ldots(2)$$

where C'_{39} and C'_{41} denote molar concentrations of $^{39}_{19}$K and $^{41}_{19}$K in the tissue. Since M is a mass large compared with that of the K atom, equation (2) may be written

$$n'_{39}/n'_{41} = C'_{39}/C'_{41}. \qquad \ldots\ldots(2a)$$

At equilibrium not only the total number of K atoms but also the ratio of the numbers of $^{39}_{19}$K and $^{41}_{19}$K atoms striking the cell membrane in the two directions must be the same. In consequence

$$\frac{C_{39}}{\sqrt{39}} \Big/ \frac{C_{41}}{\sqrt{41}} = C'_{39}/C'_{41}. \qquad \ldots\ldots(3)$$

From equation (3) the isotopic ratio of plasma K can be calculated if that of tissue K is given, and vice versa. If the value 14·20 is considered as the isotopic ratio of tissue K, the isotopic ratio of plasma K is found to be 13·85. This represents a 2·4 % increase in the $^{41}_{19}$K content of plasma K compared with that of tissue K (and mineral K), a figure which agrees well with the observed average increase of 2·5 %. The value 14·30 for tissue K would correspond to the value 13·95 for plasma K, representing a 2·3 % enrichment in $^{41}_{19}$K.

The mechanism described is proposed simply as a working hypothesis. Its claim to consideration rests on the fact that it accounts quantitatively for the observed isotope effect; while its validity depends on the condition that the intracellular K is completely associated with heavy molecules, and on the further condition that the hydration of the extracellular K ions can be neglected. If these conditions do not hold, the difference in the isotopic ratios calculable from equation (3) will be more or less reduced. Since this possibility exists, it is of interest to note that in addition to the kinetic mechanism a chemical mechanism may be in operation.

An appreciable fractionation of isotopes, due to slight differences in their chemical properties, can be obtained by means of 'exchange reactions' involving one or two phases, and a number of methods for isotope separation have been developed on this basis. A two-phase exchange reaction takes place, for instance, if the solution of an NH_4 salt is brought into contact with NH_3 gas. At equilibrium the ratios of the two N isotopes ($^{14}_{7}$N/$^{15}_{7}$N) in the liquid and gaseous phases were found to be as $\sim$1 : 1·023, thus indicating a slight enrichment of the heavy isotope in the liquid compared with the gaseous phase [Urey, 1940]. Another kind

of two-phase exchange reaction takes place if certain aluminosilicates known as zeolites, which normally contain Na as a basic constituent, are brought into contact with solutions of Li or K salts. The Na of the silicate is exchanged for Li or K, but the light isotope of Li and the heavy isotope of K are taken up preferentially. The deviations in the isotopic ratios obtained in this way were similar to the above [Taylor & Urey, 1938]. Correspondingly, the K normally present in another kind of zeolite can be exchanged for Na, the heavy K isotope being more difficult to replace than the light one [Brewer, 1939]. A certain fractionation of Li isotopes was further obtained by means of an exchange reaction between Li in alcoholic solution and Li present in Li-amalgam [Lewis & Macdonald, 1936]. Exchange reactions of these types, involving a liquid and a solid phase or two liquid phases (possibly water and lipoid), may occur in the process of K assimilation by the cell. Although the magnitude and direction of the isotope effect cannot be computed, differences approaching those observed may appear.

It remains to say that the actual values of the isotopic ratios of tissue K and plasma K do not depend solely on the mechanism responsible for their difference, but also on the isotopic ratio of the K which enters the blood stream by way of the intestine, and on the ratio of the total amount of K in tissue and plasma. Assuming that K when passing into the blood has an isotopic ratio close to 14·20, and considering the fact that the total amount of tissue K is several hundred times greater than the total amount of plasma K, the observed constancy or very slight increase in the isotopic ratio of the former and the marked decrease in the isotopic ratio of the latter are to be expected.

Summary

The isotopic constitution of K present in various rat tissues, including red blood corpuscles and blood plasma, was studied as far as the two main isotopes $^{39}_{19}$K and $^{41}_{19}$K are concerned. It was found that the isotopic ratio $^{39}_{19}$K/$^{41}_{19}$K in the ashes from bone marrow and blood plasma was distinctly lower than that of ordinary mineral K as contained in KCl (A.R.), and the content of the heavy isotope $^{41}_{19}$K was accordingly increased. K present in ash samples from other tissues showed, on the whole, the same isotopic ratio as mineral K, although a minute increase was found in exceptional cases. A kinetic mechanism has been proposed, which accounts for the difference in the isotopic ratios of tissue K and plasma K.

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16. THE BIOCHEMISTRY OF SILICIC ACID

9. ISOLATION AND IDENTIFICATION OF MINERALS IN LUNG RESIDUES AND AIR-BORNE DUSTS FROM COAL MINES

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The aetiology of the occupational lung affections described as silicosis, anthracosilicosis or more generally as pneumoconiosis is incompletely understood and a close comparison between the composition of the inhaled dusts and the minerals found in the affected lungs may be a useful preliminary to further knowledge. As most of the mineral particles in the lungs are below 5μ in diameter, identification with the petrological microscope is uncertain and X-ray diffraction apparatus becomes a necessary aid to the microscope. The qualitative knowledge of the minerals present, obtained from X-ray diffraction data, can be implemented by calculation of the minerals based on chemical or spectroscopic analysis.

It is intended in the present communication briefly to describe the methods used for the isolation of minerals from the lung tissue and for the X-ray diffraction analysis of these residues. The removal of coal and of acid-soluble material from air-borne dust from coal mines is also described, and data for a lung residue and an air-borne dust are presented. The chief minerals so far identified in this work are quartz, a member of the mica group and a member of the kaolin group.

Preparation of lung residues

Several methods have been proposed for preparing lung residues for X-ray diffraction work, and in some cases the same residues have been used for microscopic, chemical or spectroscopic analysis.

(1) *Simple drying of formalin-hardened lung tissue.* This method has been used by Sweany *et al.* [1938]: it has the advantage that the minerals are less altered than in any other method, but they are very much diluted by organic tissue and the resulting diagrams are poor (cf. Pl. 1 A).

(2) *Destruction of organic material by digestion with acid.* HNO_3 or other acid mixtures have been employed, usually followed by ignition at 600–700° [Jones, 1933; Kahane & Antoine, 1936; Jephcott *et al.* 1938; Burke & Kerr, 1938]. It gives a residue well suited for X-ray diffraction analysis, but it is always possible that minerals have been destroyed or altered by the heat treatment or by the acid attack. Burke & Kerr give evidence that even such a stable mineral as sericite shows after the treatment an X-ray diagram in which the lines are displaced against the lines of the untreated mineral. Any kaolinite present in the lung residues examined by the above investigators would have been destroyed by the heat treatment alone, as this mineral is destroyed by heating above 510°.

(3) *Destruction of organic material by enzymes.* Hicks *et al.* [1937] used this method. It is, however, slow, only part of the tissue can be removed and it is not easily applied to formalin-hardened tissue.

(4) *Removal of organic material by H_2O_2.* This method, described by Sundius *et al.* [1936] and recently used by Gärtner [1939], is in our opinion the best available, as it avoids the action of high temperatures and strong acids. As modified for the purposes of the present investigation, there are three stages in the treatment:

1. Removal of fatty material from powdered oven-dry lung with acetone.
2. Digestion with H_2O_2.
3. Extraction of the residue by acid or buffer solution.

The object of the third stage is to remove the endogenous inorganic constituents of the lung, mainly Ca, Fe, K, Na and P, as completely as possible with minimum alteration of the minerals, which form the dust in the lung. It may in some cases be impossible to attain both objectives quantitatively, but a fairly good separation can be made with repeated extraction with hot disodium hydrogen citrate at *p*H 6, or by brief treatment with cold *N* HCl. We have found that even prolonged treatment with *N* HCl or 2*N* HCl does not affect the appearance of X-ray diffraction diagrams of lung residues or mine dusts, as far as the diffraction lines of quartz or silicates of the kaolin and mica groups are concerned. That some small destruction of the quartz or more likely of the layer lattice silicates does take place is shown by the fact that SiO_2 is found in solution in the HCl extract, but this represents only a few per cent of the total silica in the dust or lung residue and would therefore not be reflected in X-ray diagrams with our present technique. Some results illustrating the extent of decomposition of mineral dusts by cold *N* HCl are shown in Table 1. With lung residues, decomposition and losses with cold *N* HCl and with hot disodium hydrogen citrate solution (*p*H 6) were slight. The amounts of SiO_2 in solution in the combined extraction and wash fluids have never exceeded a few mg., and much of this is derived from the amorphous silica of the lung tissue. The amounts of dissolved SiO_2 found in the cold HCl extracts have not been greater than those in the hot citrate extracts.

Table 1. *Treatment of powdered minerals and of air-borne dusts from coal mines with HCl*

1 g. powder shaken with 50 ml. *N* HCl at room temperature for 5 hr.

	mg. SiO_2 dissolved	Decomposition %*
Powdered minerals:		
Quartz	0·33	0·03
Kaolin	0·34	0·07
Mica sericite (Ohio)	1·81	0·45
Mica illite (S. Wales)	4·65	1·0
Air-borne dusts:		
2H2 (7·2% SiO_2)	1·26	1·8
2M1 (6·9% SiO_2)	1·22	1·8
1S1 (2·9% SiO_2)	0·48	1·7
1U1 (6·4% SiO_2)	0·55	0·9

* 100 × Dissolved SiO_2/Total SiO_2 per 1 g. powder.

The lung residues obtained by the H_2O_2 method may, however, contain large amounts of carbon (up to 80 %), and the same applies to dusts collected in coal mines. Such carbon can be removed by prolonged heating of the samples to

380° in an electric muffle. This temperature is sufficiently high to oxidize the carbon in 40–80 hr. and at the same time low enough to avoid destruction of the kaolinite lattice. Pyrites and other sulphides and hydroxides which may be present in air-borne dusts would be oxidized or dehydrated by this treatment, and they should either be centrifuged out of the dust by means of heavy liquids before heating, or identified by the X-ray diagrams of the corresponding oxides, which would be present in the heated but not in the unheated material.

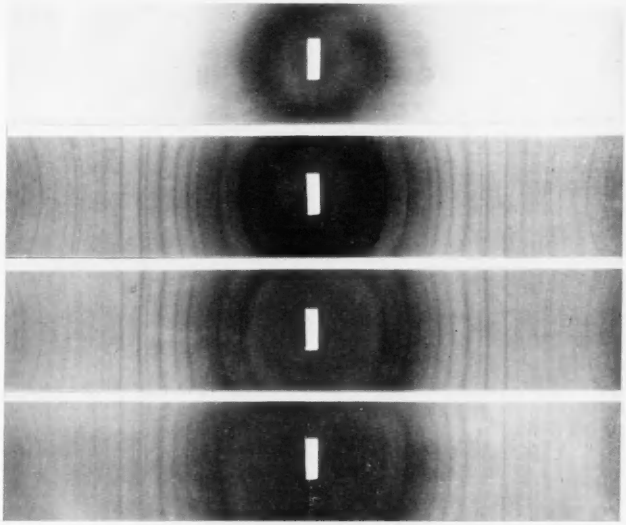
X-ray diffraction methods

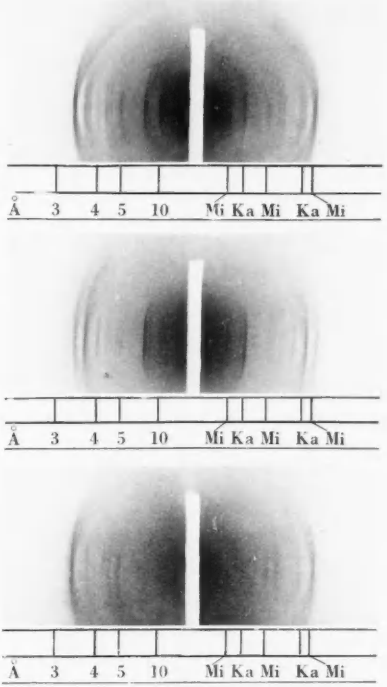
In addition to taking X-ray powder diagrams in a cylindrical camera, we have used the aggregate method which has recently been described [Nagelschmidt, 1939] in its application to soil colloids. This method is specially suitable for the identification of layer lattice silicates such as mica, kaolinite, chlorite and montmorillonite in mixtures with each other. Ordered aggregates are made by slow evaporation of a suspension of the sample to be examined, and the aggregates are mounted similarly to single crystals in an X-ray spectrometer. The particles in the aggregate are oriented with their cleavage planes parallel to the plane of sedimentation and a set of good X-ray reflexions from the basal planes is therefore obtained. These basal reflexions are the main X-ray diffraction characteristics for the different layer lattice silicates. Kaolinite has strong basal reflexions at 7·14 and 3·57 Å., and mica has strong reflexions at 10 and 3·34 Å. and a weaker one at 5 Å. When aggregates of mixtures of these two minerals are heated to 510°, only the mica reflexions remain; the kaolinite reflexions are absent, as kaolinite is destroyed by heating to 500°. Chlorite shows strong reflexions at 14·1, 7·05, 4·70 and 3·52 Å. and these reflexions are still present in aggregates heated to 510°. The aggregate method is used in addition to, but not as a substitute for, the powder method. Powder and aggregate diagrams of a lung residue and a mine dust are shown in Pl. 1.

X-ray diffraction diagrams of lung residues and mine dusts indicate qualitatively the presence of different minerals if these minerals occur in sufficient amounts. Whether and, if so, how far both the powder and aggregate methods can be developed quantitatively is at present unknown. The quantitative estimation of quartz in mine dusts has been described by Clark & Reynolds [1936], but as it is based on the intensity of the 3·34 Å. reflexion of quartz it cannot be used in the presence of mica, which gives a strong reflexion for the same spacing. The method can, however, be modified, so as to be applicable to mixtures containing quartz and mica.

Preparation details

The lung is dried at 105° and reduced to a fine powder. 20 g. of the dried substance are extracted in a Soxhlet overnight with acetone to remove fatty material. The extraction thimble with its contents is dried in an oven; the contents of the thimble are knocked into a 2 l. pyrex beaker; 30 % H_2O_2 is added to a depth of about 1 in., and the mixture is carefully warmed on a water bath and stirred with a glass rod. If the froth tends to rise to the rim of the beaker, it can be whipped down with the stirring rod and if necessary octyl alcohol is added. As the frothing subsides, and as the liquid evaporates, more H_2O_2 is added. The depth of the liquid in the beaker should not exceed about 1 in., otherwise the mixture will tend to froth over. The additions of H_2O_2, with subsequent evaporations almost to dryness, should be continued until the additions no longer cause frothing. At this point the destruction of the lung tissue seems to be complete and any black material remaining is probably coal dust.





X-Ray diffraction diagrams of a lung residue and a mine dust. A–D, Powder diagrams; E–G, aggregate diagrams. A, Oven-dry lung tissue; B, lung residue, 380° ash; C, mine dust, 380° ash after acid washing; D, mine dust, 380° ash before acid washing; E, lung residue, 380° ash; F, mine dust, 380° ash; G, same as F after heating to 520°. For reproduction, intensities in the centres of A–D have been reduced by partial shading.

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The whole process, in our experience, takes about a week. The contents are brought nearly to dryness each evening. Each morning, and as often during the day as the mixture comes to dryness, more H_2O_2 is added.

The final residue consists of the inorganic salts from the lung tissue plus the dust. The former are brought into solution by extracting the mixture with acid. We have used *N* HCl with 15 min. vigorous shaking at room temperature, and hot *M*/10 citrate buffer of *p*H 6 with continuous stirring for 1 hr. On allowing the mixture to settle, the supernatant liquid is seen to contain much of the iron in solution. Separation of the solid from the liquid can be accomplished by gravity filtration through a fine paper (a hardened filter paper seems to retain the dust particles), or by prolonged centrifuging. We have found it convenient to transfer the residue, with washing, to 250 ml. centrifuge bottles, and to use 200 ml. portions of liquid for each extraction. Each separation is carried out by centrifuging for 1 hr. at 3500 r.p.m.

The residues are re-extracted several times to remove all salts. Iron is the most difficult of the tissue constituents to eliminate from the residue, and it is usually necessary to extract the centrifuged deposits three or four times with cold HCl or hot citrate buffer. The presence of iron in the extraction fluids may be tested for by treating a sample with acetic acid, sodium acetate and ferrocyanide.

The filtrate or supernatant fluid should be checked for the absence of dust particles by microscopic examination, and by colorimetric analysis for total SiO_2 [King, 1939]. The presence of appreciable numbers of dust particles will indicate the necessity for more prolonged centrifuging. With the residues we have handled, 1 hr. spinning appears to have been sufficient. The presence of non-particulate SiO_2, i.e. dissolved SiO_2, in any quantity indicates a decomposition of the siliceous matter by the extraction fluids. The residue is finally washed once with water and once with alcohol and is dried in an air oven.

Results

As an example, we give full details for one lung residue and one mine dust. The lung was kindly supplied by Dr P. D'Arcy Hart and the mine dust was collected by Dr P. F. Holt, working with Prof. H. V. A. Briscoe. The occupational history of the case from which the lung residue was taken will be given elsewhere, in conjunction with the results obtained in the study of a large group of workers.

Lung residue. The lung residue prepared in this way lost 12% of the dry weight on prolonged heating to 380°. Powder and aggregate X-ray diffraction diagrams of the 380° ash taken with unfiltered iron K radiation are shown in Pl. 1, B and E. A powder diagram of the untreated oven-dried powdered lung tissue is shown in Pl. 1, A. Table 2 gives the interplanar spacings and intensities of diffraction lines of the 380° ash diagram, and the interpretation in terms of quartz, mica and kaolin. There are many diffraction lines of quartz which do not coincide with other lines in the diagram, but only two lines, the basal reflexions, which can be ascribed to kaolin alone. It is not possible to distinguish under these conditions which member of the kaolin group it is; it can only be said that a member of the group is present. The kaolin group contains the minerals kaolinite, nacrite, dickite, anauxite and halloysite. No other mineral is known to give strong basal reflexions at 7·1 and 3·55 Å. spacings, which disappear if the sample is heated to 510°. For the mica it is likewise not possible to determine which member of the mica group is present. The diagram does not seem to correspond to well-crystallized muscovite or hydromuscovite, but rather to the

type of mica found in shales and soil colloids. The term 'illite' [Grim *et al.* 1937] has been proposed for mica of this type, but samples described as sericite or secondary muscovite may refer to similar material.

Table 2. *X-ray data for lung residue, 380° ash*

d = interplanar spacings, Int. = intensities of diffraction lines, v.s. = very strong, s. = strong, m. = medium, w. = weak, v.w. = very weak. Q. = quartz, Mi. = a member of the mica group, Ka. = a member of the kaolin group.

} denotes edges of a band or two lines of different intensity which are not clearly separated.

No.	*d*	Int.	Mineral	No.	*d*	Int.	Mineral
1	10·1	s.	Mi.	13	1·815	s.	Q.
2	7·1	s.	Ka.	14	1·675} 1·644}	m.	Mi., Ka., Q.
3	5·0	w.	Mi.				
4	4·48}	s.	Mi., Ka.	15	1·539	m.	Q.
5	4·23}	m.	Q.	16	1·508} 1·488}	s.	Mi., Ka.
6	3·56	m.	Ka.				
7	3·35} 3·31}	v.s.	Q., Mi.	17	1·382} 1·372}	s.	Q.
8	2·58} 2·54}	s.	Mi., Ka.	18	1·294} 1·280}	w.	Mi., Ka.
9	2·46	v.w.	Mi., Q.	19	1·200	m.	Q.
10	2·45} 2·18}	v.w.	Ka., Q., Mi.	20	1·180	m.	Q.
				21	1·151	w.	Q.
11	2·131	m.	Q., Mi.	22	1·076	s.	Q.
12	2·003} 1·972}	m.	Mi., Ka., Q.				

Table 3 gives the chemical analysis of the lung residue carried out on the 380° ash. The water lost below 105° shown in Table 3 has been reabsorbed from the air by the ash. It will be seen that SiO_2, Al_2O_3, K_2O, and water above 105°, account for 94 % of the residue, and these four are the main chemical constituents of a mixture of quartz, muscovite and kaolin. It is possible to calculate the relative amounts of the three minerals in the lung residue if it is assumed that all the SiO_2, Al_2O_3 and K_2O are present in these minerals, and if the composition of the muscovite and of the kaolin are known. Such calculations with kaolin and muscovite of theoretical composition give for the lung residue: muscovite 26 %, kaolin 46 % and quartz 24 %. The chemical composition of

Table 3. *Chemical analysis of lung residue ashed at 380°*

Analyst: Chemical Laboratories, London

SiO_2	56·9
Al_2O_3	28·3
TiO_2	1·4
Fe_2O_3	1·5
MgO	0·7
CaO	0·5
Na_2O	0·8
K_2O	3·1
H_2O, above 105°	5·4
H_2O, below 105°	1·1
P_2O_5	0·1
	99·8

mica may vary within wide limits, and the mica found in shales and soil colloids usually contains only 4–6 % K_2O, as against muscovite with 11·8 % K_2O. Calculation of the minerals in the lung residue with a mica containing 5 % K_2O, 48 % SiO_2 and 32 % Al_2O_3, and kaolin of theoretical composition, gives for the

residue a mixture of 62% mica, 21% kaolin and 17% quartz. The X-ray diffraction data indicate only that all three minerals occur as main constituents. Their true relative proportions probably lie between the two sets of percentages given above. If special interest is centred on SiO_2, it will be seen that less than half of the total SiO_2 shown in the analysis, or less than a quarter of the mineral residue, occurs as quartz.

Microscopic inspection of the 380° ash of the lung residue shows that nearly all particles are well below 2μ in diameter and it does not seem possible to distinguish the different constituents quantitatively by their refractive index or other optical characteristics with microscopic petrological methods.

Mine dust. The untreated mine dust gave a poor X-ray diffraction diagram. After heating the material to constant weight at 380° a residue consisting of 15·1% of the original dust was obtained. X-ray powder and aggregate diagrams of this residue are shown in Pl. 1, D and F, and a diagram of the same residue after a short treatment with *N* HCl in Pl. 1, C. Pl. 1, G shows an aggregate diagram, taken with the same specimen as Pl. 1, F, after heating it to 540° for 24 hr. The kaolin lines are absent. Table 4 gives the evaluation of the diagram

Table 4. *X-Ray data for mine dust, 380° ash, after treatment with* N *HCl*

d = interplanar spacings, Int. = intensities of diffraction lines, v.s. = very strong, s. = strong, m. = medium, w. = weak, v.w. = very weak, Q. = quartz, Mi. = a member of the mica group, Ka. = a member of the kaolin group.

} denotes edges of a band or two lines of different intensity which are not clearly separated.

No.	*d*	Int.	Mineral	No.	*d*	Int.	Mineral
1	10·2	m.	Mi.	13	1·540	m.	Q.
2	7·1	v.s.	Ka.	14	1·508 }	m.	Mi.
3	4·48	v.s.	Ka., Mi.	15	1·493 }	s.	Ka.
4	3·55	s.	Ka.		1·484 }		
5	3·35 }	v.s.	Q., Mi.	16	1·380 }	s.	Q.
	3·31 }				1·370 }		
6	2·58 }	s.	Ka., Mi.	17	1·318	w.	?
	2·55 }			18	1·286	w.	Ka., Mi.
7	2·37 }	v.w.	Mi., Q.	19	1·255	w.	Q.
8	2·33 }	m.	Ka.	20	1·236 }	v.w.	Ka., Mi.
9	2·131	w.	Q., Mi.		1·225 }		
10	1·998 }	m.	Ka., Mi., Q.	21	1·200	m.	Q.
	1·980 }			22	1·180	m.	Q.
11	1·816	m.	Q.	23	1·151	w.	Q.
12	1·671 }	s.	Ka., Mi., Q.		1·076	m.	Q.
	1·642 }						

shown in Pl. 1, C. It is again only possible to state that quartz and members of the kaolin and mica groups are present. The presence of a line at 14·3 Å. on aggregate diagrams may possibly indicate minor amounts of chlorite. The diagram before the *N* HCl treatment shows a few additional diffraction lines indicating dolomite or ankerite. Dolomite is a calcium-magnesium carbonate and ankerite a calcium-magnesium-iron carbonate.

Table 5 gives the chemical analysis of the 380° ash of the mine dust. SiO_2, Al_2O_3, K_2O and water above 105° account for 86% of the ash and most of the remaining Fe, Ca and Mg is present as carbonate, sulphate or oxide, although a proportion of these may form chlorite with some of the SiO_2 and Al_2O_3. The mineral content has been calculated ignoring chlorite and assuming all SiO_2, Al_2O_3 and K_2O to be present as quartz, kaolin or muscovite of theoretical composition. The mine dust ash contains according to this calculation 14% muscovite, 66% kaolin and 11% quartz. Assuming a mica containing 5% K_2O,

Table 5. *Chemical analysis of mine dust ashed at 380°*

Analyst: Geochemical Laboratories, London

SiO_2	47·9
Al_2O_3	31·6
TiO_2	0·8
Fe_2O_3	3·9
MgO	1·4
CaO	3·1
Na_2O	1·0
K_2O	1·6
H_2O, above 105°	4·6
H_2O, below 105°	0·7
CO_2	0·8
SO_3	2·2
S	0·1
	99·7

48 % SiO_2 and 32 % Al_2O_3 the mine dust would contain: 32 % mica, 54 % kaolin, 8 % quartz. The water above 105° shown in Table 5 is too low to satisfy the amount of lattice hydroxyl present in kaolin of theoretical composition for either of these mixtures, but many kaolin samples contain less than the theoretical amount of water and part of the lattice hydroxyl may have been removed by the initial prolonged heating to 380°.

Free SiO_2 (quartz) [Shaw, 1934] amounted to 12 % of the mine dust ash, in fair agreement with the estimate of 11 % from the calculation with muscovite and kaolin of theoretical composition. We are grateful to Mr A. Shaw for carrying out this determination.

SUMMARY

The isolation of mineral residues from lungs by the H_2O_2 method and the removal of coal from such residues and from air-borne dusts from coal mines by heating to 380° are described. X-ray diffraction methods are used for the identification of minerals in the residues and full data, including chemical analyses, presented for a lung residue and a mine dust. Quartz, a member of the kaolin group and a member of the mica group are the main constituents found in the lung residue and in the air-borne dust.

Grateful acknowledgement is made to Dr A. Brammall for his advice and active interest in this investigation. We are indebted to the Medical Research Council for a grant covering part of the expenses of this investigation.

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17. STUDIES ON DIFFUSING FACTORS

THE HYALURONIDASE ACTIVITY OF TESTICULAR EXTRACTS, BACTERIAL CULTURE FILTRATES AND OTHER AGENTS THAT INCREASE TISSUE PERMEABILITY

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Aqueous extracts of mammalian testicle contain a factor that dramatically increases the permeability of the tissues to injected fluids [McClean, 1930; 1931; Hoffman & Duran-Reynals, 1931]. This can be demonstrated by the rapid disappearance of the bleb following intracutaneous injection of these extracts and by the rapid spread through a large area of skin of any suitable coloured indicator that may be injected together with them. This factor is associated with the germinal epithelium and can be extracted from spermatozoa [McClean, 1931]. Factors with similar diffusing properties have been obtained from the most diverse sources, for example, from filtrates and extracts of staphylococcus and streptococcus [Duran-Reynals, 1933], from organisms of the gas-gangrene group and virulent pneumococci [McClean, 1936], from extracts of malignant tissues [Duran-Reynals & Stewart, 1931; Boyland & McClean, 1935], from snake and spider venoms [Duran-Reynals, 1939] and from leeches [Claude, 1937].

Various methods for the partial purification of testicular extracts and of bacterial culture filtrates of the gas-gangrene group have been described [Morgan & McClean, 1932; Favilli, 1936; Claude & Duran-Reynals, 1937; McClean, 1936; Madinaveitia, 1938; 1939, 1, 2; Mannozzi-Torini, 1939], but no naturally occurring diffusing factor has been isolated in the pure state. Nobody had succeeded in elucidating the mechanism whereby this diffusion effect is produced until Chain & Duthie [1939] reported that purified preparations of testicular diffusing factor exhibit a remarkable mucolytic activity characterized by a rapid fall in the viscosity of mucoprotein preparations and the liberation therefrom of reducing substances. This observation suggested that the mucolytic and diffusing activities might be due to the same factor and that the spread in the tissues might be due to the action of the enzyme on the mucin-like interfibrillar substance in the collagen of the dermis.

It was obviously important to confirm the observations of Chain & Duthie, to determine whether diffusing factors from other sources exhibit a similar mucolytic activity and to make a comparative study of these enzymes. Madinaveitia & Quibell [1940] have already described a viscosimetric method whereby testicular mucinase may be accurately estimated and they have investigated many of the characters of the reaction. During the progress of our own observations Meyer *et al.* [1940, 1] reported the hydrolysis of hyaluronic acid by bacterial enzymes; it had previously been shown by Meyer & Palmer [1936] that the mucoproteins of vitreous humour and umbilical cord contain a mucopolysaccharide which they had designated by the term hyaluronic acid. More recently

Robertson *et al.* [1940] described an enzyme derived from culture filtrates of *Cl. welchii* and other organisms which hydrolyses synovial fluid; neither these authors nor Meyer and his co-workers had at that time connected the mucolytic activity of their preparations with the action on the permeability of the tissues. Subsequently, however, Meyer & Chaffee [1940] published a preliminary account of the composition of a polysaccharide obtained from the cornea and a comparison of its properties with the polysaccharides obtained from vitreous humour and synovial fluid which they consider to be identical with it and which are hydrolysed by the same enzyme preparations. They suggested that the substrate in the skin upon which the enzyme acts to produce the spreading effect may not be hyaluronic acid itself but its sulphuric acid ester. Meyer *et al.* [1940, 2] have since published a note on the relationship between 'spreading factor' and 'hyaluronidase'. They found that all preparations containing hyaluronidase also exhibit spreading properties and that both factors possess attributes in common. They have succeeded in isolating a polysaccharide from the skin which is hydrolysed by hyaluronidase. On the other hand, these authors state that a number of preparations which possess marked spreading action do not hydrolyse hyaluronic acid or reduce the viscosity of solutions containing the polysaccharide. They also reported that antisera prepared against pneumococcal hyaluronidase, which completely neutralize the hydrolytic activity, fail to inhibit the spreading activity of the pneumococcal preparations. This result is not in agreement with ours obtained with antisera prepared against the purified diffusing and hyaluronidase factors from *Cl. welchii* and *Vibrion septique*; our results will be described in the course of this paper, but it may be suggested here that the failure of Meyer and his co-workers to neutralize the spreading activity might be due to failure to balance the dose of the factor against that of the antiserum; one of us [McClean, 1936] has shown that the most potent antisera then obtainable would only neutralize a limited number of minimal diffusing doses of the spreading factor.

Favilli [1940] reported that several snake venoms which possess high diffusing activity also show a marked mucolytic effect and that this effect is inhibited by appropriate antisera. This author also reported that the diffusing extracts from leeches exhibit mucolytic activity. Claude [1937] had reported the diffusing activity in the skin of leech head extracts and he [1940] confirmed the mucolytic activity of these extracts and noted a parallelism between the mucolytic and spreading powers of various extracts. He also prepared a mucoprotein from normal rabbit skin, the viscosity of which is rapidly reduced by leech extracts, and concluded that the spreading action in the skin was due, at least in part, to the power of these extracts to cause hydrolysis or depolymerization of the same compound *in vivo*. Claude [1935] had previously reported that azoproteins diffused in the skin in a manner similar to, though with less rapidity than testicular extracts; Favilli showed that these diazotized compounds cause a marked fall in the viscosity of mucoprotein preparations, but he was unable to decide whether reducing substances were liberated as a result of this action.

Our own observations have been designed to study the characteristics of the hyaluronidase activities of extracts from testis, from various bacteria and from other sources in order to determine how far the enzymes are identical in their properties. We have also attempted to correlate the hyaluronidase and spreading activities of these preparations in order to determine whether diffusion in the tissues may be due to the action of hyaluronidase. Our observations have led us to investigate the relationship between activities of hyaluronidase and of other

enzymes such as phosphatase and amylase present in the bacterial filtrates. We have also made some preliminary observations on the part played by hyaluronidase in the metabolism of certain bacteria with a view to elucidating the activity of this enzyme in the tissues during infection. Some of the observations to be described in this paper have already been briefly reported in preliminary communications [McClean & Hale, 1940, 1, 2].

Methods

Preparation of substrate. In preliminary experiments vitreous humour was used as substrate, but, in view of its spontaneous loss of viscosity on storage, to which reference will be made later, this substance was replaced by preparations of mucoprotein from umbilical cords. The cords were stored in acetone and minced as required. 100 g. of the dried powder were extracted with 1 l. of distilled water for 24 hr. in the refrigerator and this extraction was repeated once. The fluid was squeezed through twill and both supernatants combined. The aqueous solution was purified by the method described by Meyer & Palmer [1936] which depends upon precipitation with ice-cold alcohol. We avoided the use of glacial acetic acid in this method in order to preserve the viscosity of the mucoprotein; flocculation of the precipitate was accelerated by the addition of a few ml. of saturated alcoholic sodium acetate solution. The final precipitate was washed with acetone and dried over P_2O_5 *in vacuo*. About 3 g. of a white fluffy powder can be obtained from 100 g. of dried umbilical cord. This powder contains about 7 % total N and a 0·25 % solution of it in water has a relative viscosity of about 400 compared with water as 100. Aqueous solutions were preserved by the addition of a few drops of chloroform and were kept in the refrigerator. A very slow loss of viscosity occurs. This preparation was used for most of our experiments.

For some observations, it was desired to use a purified salt of hyaluronic acid as substrate. For this purpose the preparation described above was further purified by the method of Robertson *et al.* [1940], which depends upon tryptic digestion of the protein, precipitation of this with trichloroacetic acid and final precipitation of the polysaccharide with alcohol as a potassium salt. About 350 mg. of this powder are obtained from 1 g. of the mucoprotein. 0·2 % aqueous solutions of the polysaccharide have a relative viscosity of about 350. The powder contains about 3 % total N and 0·5–1·0 % P.

Preparation and purification of enzymes. Samples of testicular enzyme purified by methods described by Morgan & McClean [1932] and by Madinaveitia [1938; 1939, 1, 2] for the purification of diffusing factor were used. Unpurified bacterial culture filtrates were employed as well as samples from *Cl. welchii* filtrates purified by the method described by McClean [1936], which depends upon precipitation of the active fraction with basic lead acetate. Filtrates from *Cl. oedematis maligni* (*Vibrion septique*) were purified with alumina C_γ by the method used by Madinaveitia [1939, 2] for *Cl. welchii* filtrates and testicular extracts. In our experience it is impossible to purify *Vibrion septique* filtrates by the lead precipitation method successfully employed with *Cl. welchii*. Our observations with Russell's viper venom were made with the dried venom in the form of 'Stypven' which was kindly supplied by Dr Parish of the Wellcome Physiological Research Laboratories.

Viscosimetric technique. Madinaveitia & Quibell [1940] have shown that, under certain defined experimental conditions, the time taken by any sample of enzyme to reduce the viscosity of the substrate to a level half-way between its original level and that of the solvent is inversely proportional to the concentration of the enzyme. They have also shown that the reaction time, that is the time taken to reach half viscosity level, is independent of the concentration of the substrate and of its original viscosity. Both these facts have been confirmed by us and, for the purpose of this work, we have provisionally defined a viscosity-reducing unit (V.R.U.) as that concentration of enzyme which will reduce the viscosity of a standard substrate preparation to a level half-way between its original figure and that of the solvent employed in 20 min. The selection of this period of time is determined by experimental conditions that will be described. Madinaveitia & Quibell[1] employed a final concentration of 0·66 *M* NaCl in their system. It appeared that the *M*/6 citrate buffer supplied sufficient electrolyte and, since we did not wish to work in a salt concentration so widely different from that present under biological conditions, we omitted the addition of NaCl in

our earlier experiments. Later, for reasons that will appear in the text, we have added NaCl to a final concentration of 0·1 *M*.

Four vol. of 0·25 % mucoprotein or 0·2 % potassium hyaluronate in distilled water are mixed with 1 vol. of *M* sodium citrate buffer at *p*H 4·6. The buffer is prepared by mixing 1 vol. of *M* citric acid with 2 vol. of *M* NaOH. The mixture of substrate and buffer is placed in the water bath at 34°. 0·5 ml. of the appropriate dilution of the enzyme is also placed in the water bath in a separate tube. A similar dilution of the enzyme previously heated for 30 min. in boiling water serves as control. Two Ostwald viscosimeters with the same or closely similar flow times are placed in the bath; these and the sample of enzyme and substrate are left to warm for at least 10 min. After this interval 2·5 ml. of the buffer substrate mixture are run into each of the tubes containing heated and unheated enzyme. A stopwatch which is started at this moment will record the reaction time (*R*). The substrate, buffer and enzyme mixtures are mixed with warm pipettes and 1·5 ml. of each are run into the viscosimeters so that one viscosimeter contains the test mixture and the other the

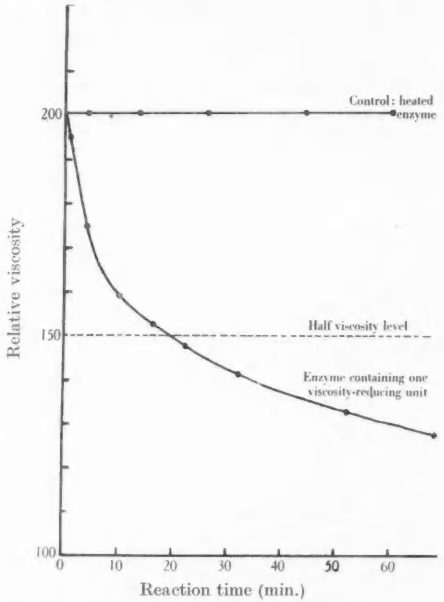

Fig. 1. Typical curve of enzyme containing one viscosity-reducing unit.

control. The fluid is blown (not sucked) into the bulb of the viscosimeter in order to avoid the formation of bubbles. The first reading is taken on another stopwatch 1 min. after mixing and thereafter at frequent intervals until the flow time of the unheated enzyme system is less than half the sum of that of the heated enzyme system and that of the solvent employed. The flow time readings on either side of the half viscosity level are plotted against time and the point at which the line crosses the half viscosity level is taken as the reaction time of the sample of enzyme under test. A correction is made for the fall in viscosity that occurs during a reading of the flow time; half the flow time is added to the time at the beginning of the run of the fluid through the viscosimeter. In order to facilitate calculation of relative viscosities, half viscosity levels and enzyme strength, all the flow times are considered to be relative to a flow time of 100 for *M*/6 sodium citrate buffer. Thus

$$\frac{\text{Flow time of substrate}}{\text{Flow time of buffer}} \times \frac{100}{1} = \text{relative viscosity.}$$

Viscosimeters were closely matched and there were not more than 1 or 2 sec. difference in the time of flow of the buffer solution. The viscosimeters used gave a flow time of about 50 sec. for 1·5 ml. of this fluid. In order to compare the potencies of different enzyme samples accurately it is necessary to use dilutions which give reaction times falling between 15 and 45 min. If the experimental conditions described are carefully observed assays of the viscosity-reducing activity of enzymes can be made with an error of $\pm 10\%$. A typical calculation of the activity of a sample of enzyme is as follows:

Reaction time to half viscosity level (R) = 30 min.
Amount of dilution of enzyme sample (D) = 60 (dilution of 1 : 60).

Then viscosity-reducing units (already defined) $= \frac{D \times 20}{R}$

$$= \frac{60 \times 20}{30}$$

$$= 40.$$

A typical viscosity reduction curve for a sample containing 1 unit of activity is shown in Fig. 1.

Estimation of reducing substances. *N*-Acetylhexosamine was estimated by the method of Morgan & Elson [1934]. The colour produced in the test solutions was matched against standard solutions in an optical colorimeter. The method gave most accurate results when the concentrations of acetylhexosamine were between 20 and 200 μg./ml. Total reducing substances were estimated by the method of Hagedorn & Jensen.

Diffusion in the dermis. Three methods of assaying diffusing activity in the skin are available. The original method described by one of us [McClean, 1930; 1931] depends upon the determination of the highest dilution of a sample that will produce a significant (20 %) increase in the area of spread in the skin. Madinaveitia [1938] has described a method which depends upon the comparison of the rate of spread of the sample under test with that of a standard preparation of diffusing factor. He did not claim that it was possible to differentiate between less than tenfold dilutions of the diffusing factor by this method. Bacharach *et al.* [1940] have recently described a method for estimating testicular diffusing factor depending upon a comparison of the areas of spread after 20 min. of two dilutions of a sample solution with those of two dilutions of a standard preparation. The injections are so arranged on the shaved backs of four rabbits as to allow for corrections for variation in animal susceptibility to the diffusing factor. These authors claim to be able to distinguish between doses in the ratio of 2 to 1. An investigation is being made into the correspondence between determinations of diffusing activity made by the method of Bacharach *et al.*, and by the original method of determining the minimal diffusing dose; observations are also in hand to determine whether both methods are equally applicable to testicular, bacterial and other diffusing substances. Pending the result of this investigation it has seemed advisable to adhere to assay by the determination of the minimal diffusing dose for the purpose of the work to be reported in this paper. Notwithstanding the fact that we cannot claim to be able to distinguish between less than tenfold dilutions, we have noticed that the dose response curve does become steeper as the limiting dilution of the diffusing factor is being reached. There is therefore less liability to confusion between the areas of spread of two succeeding dilutions than when more concentrated solutions are employed. The unsatisfactory nature of this animal test from a quantitative point of view must be admitted, but it does suffice for the broad comparison of diffusing and hyaluronidase activities attempted in this work.

Tenfold dilutions of the sample to be tested were made in isotonic haemoglobin solution. 0·4 ml. samples of these dilutions were injected into the backs of clipped or depilated white-skinned rabbits. Two control injections of 0·4 ml. of haemoglobin solution alone were made into each rabbit. The length and breadth of the areas stained with haemoglobin were measured 20 min. after injection and the areas calculated on the assumption that they were ellipses according to the formula $\pi Dd/4$ when D = the long axis and d = the short one. The assumption that the area is elliptical is valid for the higher dilutions of the spreading factor only. Estimation of the area of spread of haemoglobin is facilitated if the animal's back is smeared with liquid paraffin before the measurements are made. Our results were expressed as percentage increase in area compared with that of the control injections of haemoglobin solution alone.

Experimental

Association between the hyaluronidase activities of various extracts and their diffusion in the dermis

The mucolytic activity of testis extract reported by Chain & Duthie was confirmed with mucoprotein from either vitreous humour or umbilical cord as substrate. Preparations of this diffusing factor purified either by the method of Morgan & McClean or by those of Madinaveitia showed similar activities in the reduction of the viscosity of the substrate and the liberation of reducing substances. Our preliminary experiments were designed to demonstrate a qualitative association between the diffusing and hyaluronidase activities of enzymes from various naturally occurring sources; no quantitative comparisons of the two activities were made at this stage.

Purified preparations of the diffusing factor from filtrates of *Cl. welchii* and *Cl. oedematis maligni* (*Vibrion septique*), which in high dilution cause rapid diffusion in the skin, also show marked hyaluronidase activity as demonstrated by viscosity reduction and the liberation of *N*-acetylhexosamine. These organisms are both members of the group of gas-gangrene-producing anaerobic organisms. Filtrates from certain strains of staphylococcus, streptococcus and from a virulent type 1 pneumococcus are known to diffuse actively in the skin; as will be seen in Table 1, these filtrates also showed marked hyaluronidase activity. On the other hand filtrates from a virulent strain of *S. typhi*, from *Cl. tetani* and from *C. diphtheriae* which produce no diffusion also contain no hyaluronidase.

Table 1. *The qualitative association between diffusion in the dermis and hyaluronidase activity*

Source of enzyme	Diffusion in 20 min. Dose ml.	Diffusion in 20 min. Increase in area %	Hyaluronidase units Undiluted sample	Liberation of *N*-acetyl-hexosamine
Testis extract (bulls)	4×10^{-5}	84	90	+
Russell's viper-venom ('Stypven')	0·016	38	0·5	+
Cl. welchii filtrate (strain 107)	4×10^{-6}	35	70	+
Cl. oedematis maligni filtrate (*V. septique* KF5)	4×10^{-5}	82	22	+
Staphylococcus filtrate (strain Wood 46)	4×10^{-2}	150	60	+
Pneumococcus filtrate (type 1 virulent)	4×10^{-2}	115	6	+
Streptococcus filtrate (5 strains)	4×10^{-2}	50–100	0·3	+
Streptococcus filtrate (3 strains)	4×10^{-2}	Nil	Nil	–
C. diphtheriae filtrate (strain 110)	0·2	Nil	Nil	–
Cl. tetani filtrate	0·2	Nil	Nil	–
S. typhi filtrate	0·2	Nil	Nil	–

Note. *Diffusion tests:* 0·4 ml. of a dilution of the sample to be tested was injected intracutaneously. The area of spread of these single injections is not an accurate expression of the relative diffusing activities of the different samples and no quantitative correlation between diffusing and hyaluronidase activities was attempted in this experiment.

The activity of the filtrate from virulent type 1 pneumococci is of interest, since one of us [McClean, 1936] has reported that the same strain rendered avirulent by cultivation in type-specific immune serum loses its diffusing power; on the other hand Meyer *et al.* obtained their hyaluronidase from the autolysate of a 'non-encapsulated non-type-specific variant' of a type 2 strain and drew a distinction between the enzyme obtained from pneumococci which is bound to the cell structure and that from *Cl. welchii* which is free in the fluid medium. It is clear from our observation that encapsulated virulent pneumococci excrete the enzyme into the medium in a manner similar to that of members of the gas-gangrene group.

It has already been mentioned in the introduction that several snake and spider venoms diffuse in the skin and that Favilli had reported that such snake venoms also showed a marked mucolytic activity. Through the courtesy of Dr Parish of the Wellcome Physiological Research Laboratories we were able to examine these properties in dried Russell's viper venom ('Stypven'). The results are summarized in Table 1. It will be seen that there is again an obvious association between diffusing and hyaluronidase activities. Further observations on the activity of this venom will be described later when the general properties of these enzymes are considered.

Certain artificially prepared compounds such as azoproteins and ascorbic acid and its oxidation products were found to diffuse in the skin and to reduce the viscosity of mucoprotein. Since, however, the characters of the diffusion and of the action on mucoprotein differ in many respects from those of the naturally occurring enzymes consideration of the behaviour of these substances will be postponed until that of the enzymes has been described.

Characters of the hyaluronidase activity of enzymes from various sources

Influence of p*H*. We have confirmed the result reported by Madinaveitia & Quibell that the action of the testicular enzyme on the viscosity of the substrate is greatest in the region of *p*H 4·6. The rate of the reaction decreases sharply on the acid side of this and more gradually as the *p*H approaches neutrality. Similar observations on enzymes obtained from culture filtrates of *Cl. welchii*, *Vibrion septique* and from Russell's viper venom indicate that they are all most active in the region of *p*H 4·6. All these enzymes show the same sharp decline in activity on the acid side of this *p*H, but whereas the enzyme from viper venom is completely inactive on the alkaline side of *p*H 6·5 those from *Cl. welchii* and *Vibrion septique* still cause a slow fall of viscosity at *p*H 8·5.

Liberation of N*-acetylhexosamine by these enzymes.* Preliminary observations on the rate of liberation of *N*-acetylhexosamine from mucoprotein by these enzymes indicated that, whereas in the presence of a sufficient concentration of enzyme the fall in viscosity may be so rapid that half viscosity level is reached in a few minutes, no acetylhexosamine can be detected for some hours. An experiment was performed in which purified potassium hyaluronate was exposed to the action of the purified *welchii* enzyme with the result shown in Table 2. It will be seen that although half viscosity level was reached in between 1 and 10 min. no measurable amount of acetylhexosamine could be detected for 50 min.; the maximum liberation was not attained until considerably after 2 hr. had elapsed. Similar results were obtained with the testicular enzyme.

This result indicated that two separate mechanisms might be involved and, furthermore, our early experiments suggested that enzymes from different sources might differ qualitatively as well as quantitatively in their capacities to liberate reducing substances. Thus it appeared that the bacterial enzyme

Table 2. *Time relation of viscosity reduction and liberation of N-acetylhexosamine*

Substrate: Potassium hyaluronate 2 mg./ml.
Enzyme: Purified from *Cl. welchii* filtrate.

Time		Relative	
Hr.	Min.	viscosity	*N*-acetylhexosamine
0	0	350	0
0	1	250	0
.....			
0	10	162	0
0	20	138	Trace
0	30	127	Trace
0	50	120	<0·1 mg./ml.
1	10	115	<0·1 ,,
2	0	108	0·1 ,,
18	0	102	1·0 ,,

Viscosity of buffer solution + enzyme = 100. The dotted line indicates the half viscosity level and the approximate time taken to reach it.

liberated more reducing substances than that from testis and that no reducing substance could be detected after the action of viper venom. When, however, the concentrations of the enzymes from different sources were adjusted so that their reaction times for the reduction of viscosity were comparable, it was found that the amount of enzyme necessary to liberate measurable quantities of reducing substances was approximately the same whatever the source of the enzyme. The result of this experiment is shown in Table 3.

Table 3. *Relation between N-acetylhexosamine liberation and viscosity-reducing activity of enzymes from various sources*

Substrate: Potassium hyaluronate 1·5 mg./ml.

Source of enzyme	Dilution containing 1 viscosity-reducing unit (V.R.U.)	Amount of *N*-acetylhexosamine (mg./ml.) liberated in 18 hr. by		
		60 V.R.U.	6 V.R.U.	3 V.R.U.
Cl. welchii (strain 107)	1 : 66	1·0	0·1	Trace
Staphylococcus (Wood 46)	1 : 60	0·8	0·1	Trace
Pneumococcus (type 1 virulent)	1 : 6·6	—	Trace	Trace
Russell's viper venom ('Stypven' 2 mg./ml.)	1 : 10	—	0·1	0·1
Testis (Madinaveitia standard prep. 10 mg./ml.)	1 : 5	—	0·4	0·2

Later observations indicated that there is not complete correlation between viscosity reduction and liberation of reducing substances by different samples of enzyme from the same source. Thus, in the experiment illustrated in Table 3, the testicular enzyme liberated at least four times as much acetylhexosamine as comparable concentrations of enzymes derived from bacteria and venom; in another experiment, with a sample of testicular enzyme purified by a different method, it was found to liberate less than half the amount of acetylhexosamine liberated by a *welchii* enzyme of the same viscosity-reducing activity. The time lag in the liberation of reducing substances, coupled with the lack of complete correlation between this activity and the reduction of viscosity, suggest that two separate mechanisms may be involved; if the enzymes are not identical, they

must be very closely associated since they are not separated by any existing method of purification and, as will appear, they are similarly influenced by variations in the environment of the reaction.

Neutralization of enzyme activity by antisera. One of us [McClean, 1936] has already reported that antisera can be prepared which inhibit the spread of diffusing factors of bacterial origin in the dermis; it was also reported that the animals actively immunized against the purified diffusing factor in order to produce these antisera were themselves resistant to the diffusing activity of the homologous factor. Experiments were made to determine whether these sera

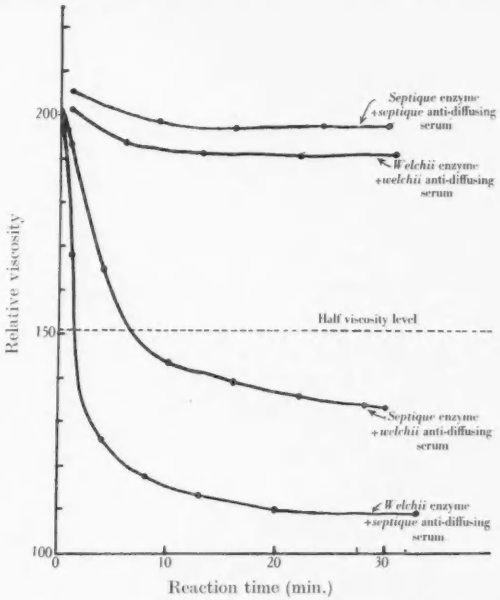

Fig. 2. The neutralization of hyaluronidases by anti-diffusing sera.

Table 4. *The influence of anti-diffusing sera on the liberation of N-acetylhexosamine*

Substrate: Vitreous humour.
Enzyme: Prepared from culture filtrate of *Cl. oedematis maligni* (*Vibrion septique*).

Serum	Liberation of *N*-acetylhexosamine (mg./ml.) in 18 hr. by falling dilutions of enzyme from *Vibrion septique*		
	Undiluted	1 : 10	1 : 100
Vibrion septique anti-diffusing serum	Nil	Nil	Nil
Cl. welchii anti-diffusing serum	1·0	0·1	Trace
Normal rabbit serum	1·0	0·1	Trace
Saline	1·0	0·1	Trace
Heated enzyme + saline + vitreous humour	Nil	—	—

inhibited the viscosity-reducing activity of the bacterial enzymes and the liberation of reducing substances by them. The influence on viscosity reduction is shown in Fig. 2 and on liberation of reducing substances in Table 4. Similar experiments on the inhibition of the activity of the testicular enzyme could not be undertaken since the purified enzyme from this source has never been obtained in an antigenic form.

Equal volumes of undiluted serum and the enzyme preparations were mixed and left at room temperature for 30 min. before addition to the substrate. In the observation on viscosity-reducing activity both *Cl. welchii* and *Vibrion septique* enzymes were set up with the homologous and heterologous antisera using a substrate of mucoprotein derived from umbilical cord. In the observation on the liberation of acetylhexosamine the enzyme from *Vibrion septique* was the only one used; it was set up against the homologous antiserum, against a *welchii* antiserum, normal rabbit serum and saline. The amount of acetylhexosamine liberated was estimated after 18 hr. incubation at 37°. It will be seen that in both experiments the inhibition of enzyme activity was complete and strictly specific; the heterologous antisera and normal serum exerted no effect. The diffusing activity of these enzymes in the skin occurs at a much higher dilution than that at which any *in vitro* viscosity-reducing activity can be demonstrated. It has already been shown [McClean, 1936] that these antisera can only completely inhibit a limited number of minimal diffusing doses of diffusing factor in the skin and this probably explains the failure of Meyer *et al.* [1940, 2] to demonstrate inhibition of diffusion with pneumococcal antiserum.

Influence of electrolytes on the reaction. Madinaveitia & Quibell [1940] employed a final concentration of 0·66 *M* NaCl and 0·166 *M* sodium citrate in their viscosimetric tests. For reasons given in the section describing our methods, we omitted the NaCl from our system, but retained the 0·166 *M* sodium citrate buffer. A comparison of estimations of the activities of several samples of both testicular and bacterial enzymes revealed a gross disparity between us and led to the investigation of the influence of electrolytes on this reaction.

The apparent viscosity-reducing activities of a purified testicular enzyme and of purified enzymes derived from *Cl. welchii* and *Vibrion septique* were compared at concentrations of NaCl varying from nil up to *M*. The concentration of sodium citrate at 0·166 *M* was kept constant throughout the experiment. The influence of varying concentration on the initial viscosity of the substrate was controlled in all titrations of enzyme activity. The result of this experiment is shown in Fig. 3 in which the apparent strength of the enzyme is expressed in viscosity-reducing units plotted against NaCl concentration.

It will be seen that there is a marked retarding effect on enzyme activity as the salt concentration is increased above 0·3 *M*, but that below this level there is an apparent difference between the behaviours of testicular and bacterial enzymes; the testicular enzyme shows a rapid apparent fall in activity as the salt concentration is reduced to nil, whereas the activity of the bacterial enzymes appears to be maintained at a high level. Madinaveitia (personal communication) has shown that in salt concentrations below 0·1 *M* the direct relationship between enzyme concentration and reaction time on viscosity breaks down and that this lack of correlation is more marked for weak enzyme preparations. Since the method of estimating enzyme activity depends upon the employment of a dilution of the enzyme which will give a reaction time of 15–45 min. it is possible that considerable errors may develop in the assay of activity at very low salt concentrations. Thus it appeared that the difference between the behaviours of testicular and bacterial enzymes might be more apparent than real. Further-

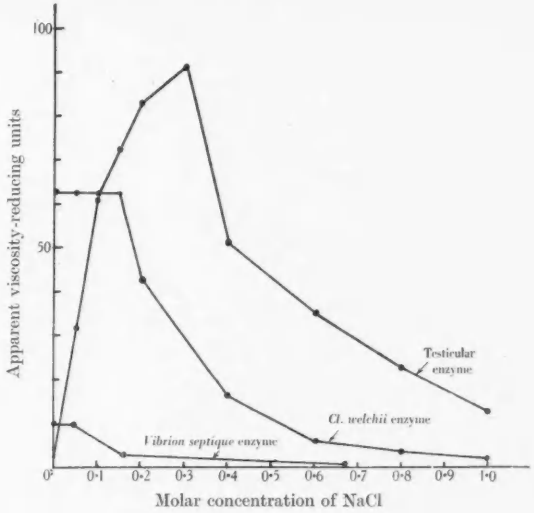

Fig. 3. The influence of NaCl concentration on the viscosity-reducing activity of testicular and bacterial enzymes.

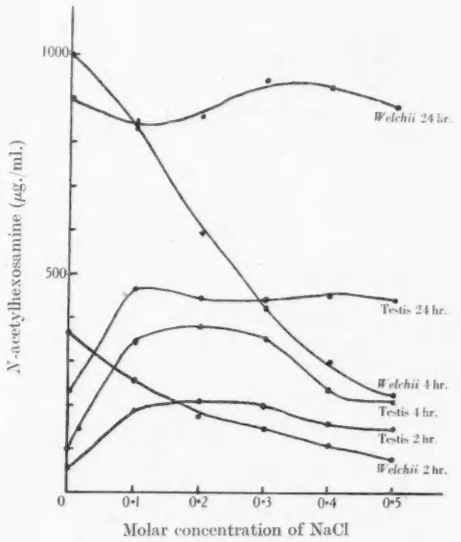

Fig. 4. The influence of NaCl concentration on the rate of liberation of *N*-acetylhexosamine by testicular and bacterial enzymes.

more, the purified bacterial enzymes were derived from culture filtrates and might contain small amounts of salts or other impurities sufficient to maintain a high level of activity. It should be stated, however, that both the testicular and bacterial enzymes had been exposed to prolonged dialysis during purification, were of considerable potency and were therefore used in dilutions which should have sufficed to eliminate the influence of impurities.

In order to obtain confirmatory evidence of the influence of salt concentration on the rate of the reaction, an experiment was performed to compare the rates of liberation of acetylhexosamine by testicular and *welchii* enzymes in the presence of NaCl concentrations ranging from nil to 0·5 *M*. The amount of acetylhexosamine was estimated after 2, 4 and 20 hr. incubation at 37°. The result is shown in Fig. 4.

In this experiment ninety viscosity-reducing units of each enzyme were used; the enzymes were therefore much more concentrated than in the foregoing experiment and there is no question of direct proportionality between reaction time and enzyme concentration. It will be seen that after 2 and 4 hr. the maximum liberation of acetylhexosamine by the *welchii* enzyme has occurred at the lowest NaCl concentrations and that there is a steady fall as the salt is increased. The testicular enzyme, however, shows an increase in activity up to 0·2 or 0·3 *M* NaCl, similar to that which appears in the experiments on viscosity reduction; there is a fall in the yield as the salt concentration is further increased. There is no significant difference in the amount of acetylhexosamine liberated by either enzyme at any salt concentration after 24 hr.; the reaction is then presumably complete. Both activities appear to be similarly affected by variations in salt concentration and the difference between the viscosity-reducing activities of testicular and bacterial enzymes is confirmed in respect of the liberation of acetylhexosamine. These experiments show that the potency assigned to enzyme preparations will be profoundly affected by the NaCl concentration of the system and this has some bearing on the correlation between diffusing activity in the skin, which presumably occurs in isotonic salt, and *in vitro* hydrolysis of the substrate; this point will be considered later.

The part played by the different ions in the system was investigated. In order to determine the part played by the Na ion the concentration of sodium citrate at *p*H 4·6 was increased by amounts calculated to give a weight of Na equivalent to the various amounts of Na present as NaCl plus the constant amount of sodium citrate employed in the previous test. The activities of both testicular and bacterial enzymes at various sodium levels were estimated viscosimetrically in the normal way, allowance being made for the influences of sodium citrate concentrations on the initial viscosity of the system. The results showed that increase in Na concentration exaggerated the depressing effect on enzyme activity and almost eliminated the initial rise in the apparent activity of testicular enzyme which occurs in the presence of small concentrations of NaCl.

In order to vary the concentrations of the Cl ion in the presence of a constant amount of Na as citrate an experiment was set up in which the usual concentration of *M*/6 buffer was used in the presence of increasing concentrations of NH_4Cl. The result with both enzymes was in every way comparable with the action of Cl as NaCl.

A further experiment was performed on the influence of increasing concentrations of Na_2SO_4 in the presence of the constant amount of citrate buffer. The result obtained was the same as that with increasing amounts of sodium citrate.

These observations indicate that the accelerating action on both enzymes at low salt concentration is due to the Cl ion and that the Na ion exerts a depressing

effect at similar concentrations. As the amount of salt is increased beyond a certain point both ions exert depressant influences. A preliminary account of these investigations together with those made by Madinaveitia has been published [Madinaveitia & McClean, 1940], and he has shown that the intensity of the apparent accelerating effect is in the order $Cl > Br > I > SO_4$ and the retarding effect at higher concentrations the opposite.

Relation of viscosity to concentration of substrate and the influence on enzymic activity. Madinaveitia & Quibell [1940] reported that the reaction constant and the half-life time of the viscous substrate are constant for a fixed amount of enzyme regardless of the concentration of the substrate. Robertson *et al.* [1940], on the other hand, found that the time taken for a fixed amount of enzyme to break down increasing amounts of substrate was proportional to the concentration of the latter. We have found that the reaction time of the enzyme is constant in concentrations of substrate varying from 0·66 to 6·6 mg./ml., thus confirming the findings of Madinaveitia and Quibell over a much greater range of viscous substrate than that of their experiment. In order to clear up this discrepancy we were led to investigate the relation of the viscosity to concentration of the substrate and the influence of NaCl on this relationship; we also wished to find out whether it is justifiable to use viscosity as a direct measurement of the concentration of mucous material or to assume that the half-viscosity level is that at which half of the substrate has been rendered non-viscous.

The viscosity of mucoprotein at concentrations increasing from 0·66 to 6·6 mg./ml., in the whole system as set up for enzyme titrations, was determined both in the absence of salt and in the presence of 0·66 *M* NaCl. The results are shown in Fig. 5.

It will be seen that the viscosity increases at a much greater rate than the concentration of the substrate and that this effect is more marked as the concentration increases. Therefore the reduction of viscosity by half does not indicate that half the substrate has been rendered non-viscous. If we assume that when half-viscosity level is reached the substrate consists of a mixture of completely viscous and completely non-viscous material, the following conclusions can be drawn from these curves: as the concentration of the substrate is raised a gradually increasing amount but decreasing proportion of the total weight of substrate is rendered non-viscous when half-viscosity level is reached. Whether NaCl is present or not, the proportion of substrate rendered non-viscous at this level is approximately the same; therefore we think it is justifiable to assume that the half-viscosity level at different NaCl concentrations does represent a comparable decrease in the concentration of viscous material, but it is not true to say that the percentage decrease in the concentration is independent of the original concentration of mucoprotein. As already confirmed by us, however, it is an experimental fact that the reaction time of a sample of enzyme remains constant and is completely independent of the concentration of the substrate. Since the proportion of substrate destroyed by the enzyme is not constant in different concentrations of substrate this raises interesting questions as to how the constant reaction time is attained. It may be that a balance is established between increased rapidity in the formation of enzyme-substrate complex as the concentration is raised and slowing of molecular movement due to the increased viscosity of the system.

These observations on the relation between viscosity and concentration of substrate provide a possible explanation for the apparent discrepancy between Madinaveitia & Quibell's findings that the reaction time is constant regardless of the concentration of substrate and the report by Robertson *et al.*, that the rate of

reaction is proportional to the concentration. The latter workers took their reactions to an end-point when either the viscosity was completely destroyed or the precipitability of the mucoprotein was abolished. From the curves in Fig. 5 it can be deduced that if the original substrate contains 6·6 mg./ml. then, when half-viscosity level is reached, only 1·45 mg./ml. will have been destroyed and 5·15 mg./ml. will remain. If, however, the original substrate only contains

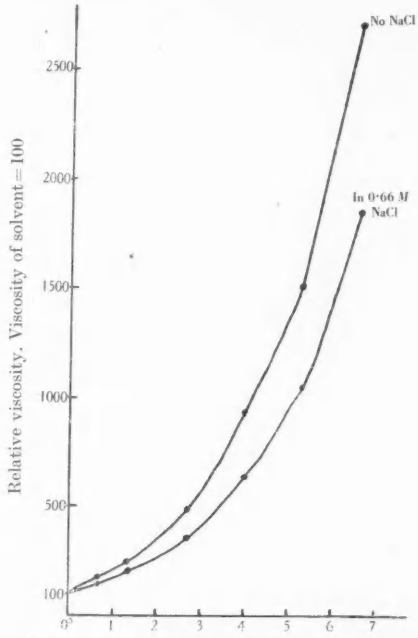

Fig. 5. The relation of viscosity to concentration of mucoprotein.

0·66 mg./ml., then at half-viscosity level 0·30 mg./ml. will have been destroyed and only 0·32 mg./ml. remain. Therefore the latter reaction, at the lower initial concentration, will probably be carried to completion more quickly, although the time taken to reach half-viscosity level is the same with both samples of substrate.

Relation of hyaluronidase activity to amylase and phosphatase, and to the action on other polysaccharides

Robertson *et al.* [1940] stated that mucinase causes a loss of viscosity of starch paste and that in some respects mucolytic and amylolytic activities are similar. They also stated that the first stage in the breakdown of mucin is catalysed by serum phosphatase. It seemed desirable, therefore, to investigate the amylolytic and phosphatase activities of our testicular and bacterial hyaluronidase preparations in order to determine whether there is any correlation between these activities.

Pastes were prepared from rice, maize, potato and wheat starches according to the method described by Waldschmidt-Leitz & Mayer [1935] except that the concentration of starch employed was slightly less than half that used by these authors. In the experiments with testicular and bacterial enzymes the proportions of enzyme and of sodium citrate buffer to the starch substrate were the same as those used in the determination of hyaluronidase activity with mucoprotein substrate.

Preliminary observations indicated that testicular enzyme did not reduce the viscosity of any of these starch pastes and that purified enzymes derived from *Cl. welchii* and *Vibrion septique* reduced the viscosity of both potato and wheat starch, but that the rate of the reaction bore no relation to their hyaluronidase activities.

An experiment, the results of which are shown in Table 5, was performed to compare the amylase and hyaluronidase activities of several bacterial and testicular preparations and to determine whether an increase in NaCl concentration exerts an effect on the amylase activity similar to that on the hyaluronidase.

Table 5. *A comparison of the amylase and hyaluronidase activities of various enzyme preparations*

Source of enzyme	NaCl concentration	Amylase activity. Viscosity reduction	Hyaluronidase activity. Viscosity-reducing units
V. septique A	Nil	30 min. to reach H.V.L.	13
,, B	Nil	35 min. to reach H.V.L.	3·5
,, B	0·1 *M*	2 hr. 14 min. to reach H.V.L.	—
,, B	0·2 *M*	2 hr. 50 min. to reach H.V.L.	—
,, C	Nil	No activity	10
Cl. welchii A	Nil	392–334 in 1 hr. 14 min. (H.V.L. = 246)	55
,, B	Nil	354–340 in 1 hr. 35 min. (H.V.L. = 227) H.V.L. not reached in 21 hr.	120
Testis	Nil	No activity	2·5
,,	0·3 *M*	No activity	91

H.V.L. = Half-viscosity level.

Viscosity figures refer to relative viscosity. Buffer + heated enzyme = 100.

It will be seen that there is no correlation between the hyaluronidase and amylase activities of these preparations and that the addition of NaCl up to a final concentration of 0·3 *M* does not produce any amylolytic activity in the testicular enzyme, in striking contrast to its marked enhancing action on the hyaluronidase activity of the same specimen. The addition of NaCl produces a retarding effect on amylolytic activity of the *Vibrion septique* preparation similar to that produced on the hyaluronidase activity of bacterial enzymes.

When the starch pastes used were hydrolysed with *N*/10 H_2SO_4 a positive Benedict reaction was produced after boiling for 15 min. No Benedict reaction could be obtained even after 24 hr. incubation with the bacterial enzymes notwithstanding the substantial fall in viscosity that had occurred; the iodine reaction also remained positive. Takadiastase, which contains no hyaluronidase and does not diffuse in the skin, causes a rapid fall in the viscosity of starch paste with the liberation of reducing sugars.

We are indebted to Dr King of the Post-Graduate Medical School, London, for the phosphatase estimations on samples of hyaluronidase from various sources. The method employed has been described by King [1939]; disodium phenyl phosphate was employed as substrate for the phosphatase estimations and diphenyl phosphate for the estimations of phosphodiesterase.

Table 6. *A comparison between the hyaluronidase, phosphatase and phosphodiesterase activities of various enzyme preparations*

Source of enzyme	Hyaluronidase activity. Viscosity-reducing units	Primary phosphatase units		Phosphodiesterase units	
		*p*H 4·5	*p*H 10	*p*H 4·5	*p*H 10
Testis	10	3·0	1·8	Nil	3
Cl. welchii A	118	4·2	Nil	Nil	1
,, B	54	2·1	—	Nil	—
V. septique A	8	—	Nil	—	—
,, B	20	3·4	—	Nil	—
Viper venom A	10	—	6·4	—	Nil
,, B	3	2·1	—	Nil	—
S. typhi	Nil	25	Nil	Nil	Nil
Takadiastase (1%)	Nil	68	—	Nil	—

It will be seen that two samples of enzyme from *Cl. welchii* purified by different methods and of widely different hyaluronidase activities are included in the list and that samples of viper venom were tested in addition to the testicular and bacterial enzymes. A filtrate from a culture of *S. typhi* was included as an example of a bacterial filtrate containing no hyaluronidase and a solution of takadiastase as a control substance known to contain a phosphatase active at *p*H 4·6. These estimations indicate that, when the above-mentioned phosphoric esters are employed, there is no evidence of any correlation between the phosphatase or phosphodiesterase and the hyaluronidase contents of any of these samples; the phosphatase activities of all the samples were very slight. It appears that amylase and phosphatase play no part in the hyaluronidase activity of any of these enzyme preparations.

Neither the testicular nor bacterial enzymes exerted any action on the viscosity of gum acacia or that of the bacterial polysaccharide isolated from *Bact. dysenteriae* (Shiga) by Morgan & Partridge [1940].

Robertson *et al.* [1940] state that mucinase derived from *Cl. welchii* does not affect gastric mucin (human or swine), but Meyer *et al.* [1940, 1] report that whereas the enzymes from pneumococcus and streptococcus have no action on the neutral polysaccharide or mucoitin-sulphuric acid from pig gastric mucosa, the enzyme from *Cl. welchii* does hydrolyse these substrates. We tested the viscosity-reducing activity of hyaluronidase from testis, streptococci, *Vibrion septique* and *Cl. welchii* on a 1% solution of commercial gastric mucin (granular mucin, type 1701 W. Wilson Laboratories), with the results presented in Table 7. In addition we performed an experiment similar to that to be described in the section on the part played by hyaluronidase in bacterial metabolism: *Cl. welchii* was cultivated in a basal medium alone and with the addition of glucose, potassium hyaluronate or gastric mucin. As will be seen in the second part of Table 7, the addition of glucose causes a slight increase and the addition of hyaluronate a considerable increase in the production of hyaluronidase. The inclusion of gastric mucin results in the production of less hyaluronidase than in the culture containing glucose, but the action of this filtrate on gastric mucin shows considerable enhancement.

Our results show no correlation between hyaluronidase activity and the action on gastric mucin. Whereas testicular and streptococcal enzymes of considerable hyaluronidase potency have no action on gastric mucin, a weak hyaluronidase from *Vibrion septique* caused a slight fall in the viscosity of gastric

Table 7. *The relationship between hyaluronidase and an enzyme which hydrolyses mucin from pig gastric mucosa*

Source of enzymes	Hyaluronidase. Viscosity-reducing units. *p*H 4·6	Fall in viscosity of gastric mucin in 2 hr. (undiluted sample of enzyme). *p*H 7·0
I. Testis extract (no NaCl)	2·5	No activity
Testis extract (in 0·3 *M* NaCl)	90	No activity
Streptococcus filtrate	330	No activity
Vibrion septique filtrate	10	292–272 (7%)
II. *Cl. welchii* filtrate. Medium:		
A. 3·5% Evans peptone alone	15	268–256 (7%)
B. 3·5% Evans peptone + 0·5% glucose	180	268–237 (18%)
C. 3·5% Evans peptone + 0·5% potassium hyaluronate	590	268–246 (13%)
D. 3·5% Evans peptone + 0·5% gastric mucin	77	268–213 (33%)

Note. No action on gastric mucin by any of these enzymes could be detected at *p*H 4·6.

mucin. *Cl. welchii* enzymes of widely different hyaluronidase activities possessed slight actions on gastric mucin which were not proportional to their activities on umbilical mucoprotein. The action of *Cl. welchii* on gastric mucin is, however, exalted by cultivation in the presence of this substance. Culture filtrates from *Vibrion septique* and *Cl. welchii* both liberate reducing substances from gastric mucin.

Relation of hyaluronidase in viper venom to its coagulant action on blood

Russell's viper venom which diffuses in the dermis also causes a rapid fall in the viscosity of mucoprotein with the liberation of reducing substances. This venom promotes the coagulation of haemophilic blood, but its mode of action has not been completely explained. Since the chemical constitution of heparin is closely allied to that of hyaluronic acid, it was thought that the hyaluronidase in the venom might destroy the heparin present in the circulating blood and thus accelerate coagulation. It was, therefore, of interest to determine whether this venom and the hyaluronidase from testis and *Cl. welchii* exert any destructive action on heparin and whether they accelerate the coagulation of blood.

Several experiments were performed in which plasma, to which sufficient heparin had been added to delay coagulation at 37° for 1–2 hr., was mixed with varying amounts of viper venom and with testicular and bacterial enzyme preparations. These observations were made with heparinized plasma alone and in the presence of 0·1% lecithin which itself reduces the anticoagulant action of heparin. Control tests were made with the addition of varying amounts of brain pulp as a source of thrombokinase to the heparinized plasma; this pulp induced rapid coagulation. No evidence was obtained from many experiments that viper venom, testicular or *Cl. welchii* enzymes reduce the anticoagulant action of heparin or that they act in a manner comparable with thrombokinase.

A further experiment was performed to determine whether viper venom, testicular or bacterial enzymes exert any direct destructive action on heparin in the absence of plasma. Heated and unheated samples of these enzymes were mixed with constant volumes of a heparin solution of known strength and the mixtures were incubated for 18 hr. at 37°. After incubation the enzymes were inactivated by boiling for 30 min. and, after cooling, a constant volume of blood was added to each tube. There was no reduction of clotting time in those tubes in

which the heparin had been exposed to the various enzyme preparations; it can therefore be concluded that the heparin molecule was still intact and these observations confirm and extend the statement by Meyer *et al.* [1940, 1] that the enzymes obtained by them from pneumococci and *Cl. welchii* do not hydrolyse heparin. In view of the uniformly negative results of all our experiments they are not presented in detail, but we wish to record our thanks to Dr R. G. MacFarlane of the Wellcome Physiological Research Laboratories for supplies of lecithin emulsion and for much invaluable advice on the arrangement and interpretation of these experiments.

Artificial diffusing factors; influence on the viscosity of mucoprotein and diffusion in the dermis

Claude [1935] reported that azoproteins obtained by coupling diazobenzenesulphonic acid with serum, albumin, casein or gelatin diffuse in the skin in a manner comparable with testicular extract. Favilli [1940] reported that these azoproteins, diazobenzenesulphonic acid and its sodium salt reduce the viscosity of synovial fluid, but he encountered difficulties in deciding whether reducing substances are liberated as a result of this action. We have confirmed Favilli's observation that diazotized compounds reduce the viscosity of mucoprotein and that the fall in viscosity is slow compared with that produced by naturally occurring enzymes. The relative slowness of this reaction is of interest, since it is comparable with the rate of diffusion of these compounds in the skin; the greatest diffusion of naturally occurring enzymes takes place in the first 20 min. after injection, while that produced by diazotized compounds develops slowly, is not obvious for an hour or more and attains its maximum after 9 hr.

We have overcome the difficulties which arise in determining whether reducing substances are liberated. These difficulties are due to the coloured nature of the diazotized compounds, their own reducing power and the difficulty of removing soluble protein. After the azoprotein had been incubated with the mucoprotein substrate for 18 hr., attempts were made to remove the protein by precipitation with ammonium sulphate or with neutral lead acetate. Neither of these methods was satisfactory; treatment with ammonium sulphate resulted in a heavy precipitation when Ehrlich's reagent was added in the estimation of acetylhexosamine and, after treatment with neutral lead acetate it was impossible to remove the lead with H_2S in the usual manner. It was found, however, that if basic lead acetate (B.D.H.) is added to the mixture of azoprotein and substrate, to which sufficient NaOH has already been added to produce a deep red colour, the protein is precipitated and the residual lead can be removed with H_2S; the latter gas is removed by bubbling air through the mixture. The efficacy of this method was tested on samples to which a known quantity of acetylglucosamine had been added and also on mixtures of substrate with natural enzyme in which reducing substances had been liberated. Our observations were also controlled by tests on mixtures of the substrate with normal serum and with water and on the substrate alone. When acetylglucosamine is estimated in the usual manner by Morgan & Elson's method a yellow colour develops on the addition of Erhlich's reagent which alters the final colour slightly, but this can be compensated for by blending with the contents of known positive and negative tubes. Total reducing sugars can be estimated by the method of Hagedorn & Jensen after this lead treatment. Our observations indicate that after the viscosity of mucoprotein has been reduced by the action of diazotized compounds no increase of reducing substances can be detected; there is no evidence that diazotized compounds destroy reducing substances already present in the mixture.

As already explained in the section describing our methods, vitreous humour was replaced by mucoprotein from umbilical cord as substrate in our observations because of the spontaneous loss of viscosity by the former substance. Robertson *et al.* [1940] suggested that this loss of viscosity is due to traces of ascorbic acid and stated that the first stage in the breakdown of mucin is catalysed rapidly by 'ascorbic acid-peroxide'. It was of interest to investigate the nature of this reaction and to determine whether ascorbic acid and its oxidation products show any diffusing action in the skin.

Ascorbic acid was dissolved in freshly boiled distilled water at $M/5$ and $M/50$ concentrations. Samples of each concentration were taken, the first being treated with one molecular equivalent of H_2O_2, the second with two molecular equivalents and the third not treated. These specimens were tested for their action on the viscosity of the usual mucoprotein substrate buffered at *p*H 4·6. The fall in viscosity caused by all three specimens at $M/50$ concentration was extremely rapid, but it appeared that the sample containing one equivalent of H_2O_2 was slightly more active than the other two. The test was repeated with $M/500$ solutions, giving a final concentration of $M/3000$ in the system, and the result is shown in Fig. 6. It will be seen that the ascorbic acid and its oxidation

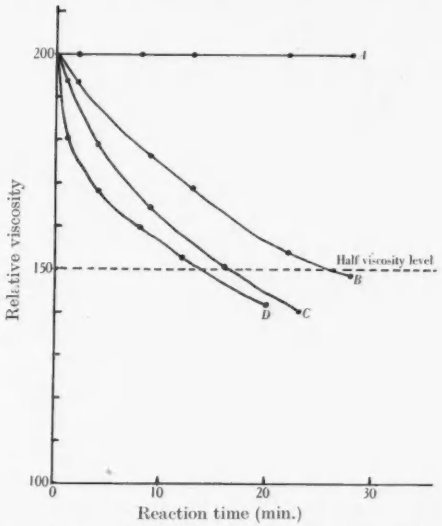

Fig. 6. The reduction of viscosity by ascorbic acid derivatives. A = control, water only. $B = M/3000$ ascorbic acid + two equivalents of H_2O_2. $C = M/3000$ ascorbic acid alone. $D = M/3000$ ascorbic acid + one equivalent of H_2O_2.

products all cause a rapid fall in viscosity, but that the specimen with one equivalent is slightly more active than the other two, half-viscosity level being reached in less than 15 min.

In order to test the diffusing activity of these preparations in the dermis it was necessary to buffer them at *p*H 7·0 before injection. The results of these diffusion tests are shown in Fig. 7. The diffusion of all three preparations in the skin is marked but develops slowly, attaining a maximum after 3 hr., and is of the same type as the spread due to diazotized compounds; a curve illustrating

the typical rapid diffusion of testicular enzyme is included in the figure for comparison. The preparation with one equivalent of H_2O_2 spread over a greater area than either of the others.

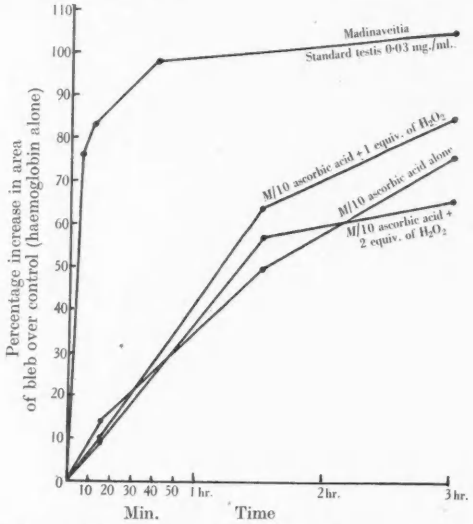

Fig. 7. The diffusion of ascorbic acid derivatives compared with that of testis diffusing factor.

It was thought that the action of ascorbic acid and its products on the viscosity of mucoprotein might be reversible. Samples of the substrate were taken after 40 min. and 18 hr. exposure to the dehydroascorbic acid, together with controls of substrate with water, and these were dialysed for 48 hr. The viscosity of the dialysed material was measured, but no evidence was obtained of any recovery of viscosity.

Notwithstanding the extremely rapid fall in viscosity of the substrate, no detectable acetylhexosamine is liberated by ascorbic acid or its derivatives; in this respect the action resembles that of the diazotized compounds. The addition of enzyme obtained from *Cl. welchii* to substrate which has already been exposed to ascorbic acid oxidation products results in the liberation of the normal amount of acetylhexosamine.

The characteristics of the action of these preparations on mucoprotein and their diffusion in the skin differ in several respects from that of naturally occurring hyaluronidase. The failure to liberate acetylhexosamine and the slow diffusion in the skin has already been noted. In addition we observed that the reaction time measured viscosimetrically is not proportional to the concentration of ascorbic acid or its products that are present. The increase in reaction time as the dilution is increased from $M/5$ to $M/500$ is very small. The reaction time is not independent of the concentration of substrate as it is with the naturally occurring enzymes; increase in this concentration causes a marked lengthening of the reaction time. The dermal diffusion of naturally occurring enzymes is evident at a much higher dilution than that at which reduction of viscosity of mucoprotein can be detected; on the other hand, the viscosity-

reducing activity of ascorbic acid and its derivatives can be detected in dilutions much greater than those necessary to demonstrate dermal diffusion.

An accidental observation in the course of some control tests revealed the fact that 0·0066 *M* thiolacetic acid reduces the viscosity of mucoprotein and that this reduction passes the half-viscosity level during 18 hr. incubation. This led us to investigate whether other reducing substances, such as H_2S, quinol, pyrogallol, glucose, sodium sulphite and 'metol', had similar actions and whether they showed any diffusing action in the skin. Tests were made to exclude the possibility that these substances activated an enzyme already present in the substrate and to determine whether reducing substances were liberated as a result of the action on viscosity. All the above substances, with the exception of glucose, cause a similar reduction of viscosity of the substrate and none of them liberate reducing substances; the viscosity of heated substrate is reduced, which excludes the possibility that an enzyme already present is activated by these agents. Several of these substances produce definite diffusion in the skin which is of the slow type similar to that caused by diazotized compounds and ascorbic acid. This activity and its relation to the reduction potential of the various substances is being investigated and the results will be described in a later communication; it is hoped that it may help to elucidate the mode of action of diazotized compounds and ascorbic acid.

The influence of hyaluronidase on bacterial metabolism

Preliminary experiments have been made to determine whether bacteria which produce hyaluronidase can use hyaluronic acid as a metabolite if it is included in the culture medium and what influence the presence of this substance has upon the production of the enzyme by the organism.

A rapidly growing 2 hr. culture of *Cl. welchii* was washed in saline to remove any hyaluronidase present in the culture fluid; the washed suspension was used as inoculum for tubes containing the following four media:

(*a*) A basal medium of 3·5 % Evans peptone alone.

(*b*) 3·5 % Evans peptone + 0·5 % glucose.

(*c*) 3·5 % Evans peptone + 0·5 % potassium hyaluronate.

(*d*) The same composition as (*c*) with the addition of 1 unit of hyaluronidase to start the enzymic action on the hyaluronate.

Anaerobiosis was maintained in these tubes by the addition of 1 % by vol. of a 2 *M* thiolacetic acid in *N* HCl; the *p*H was corrected by neutralization with NaOH. The cultures were incubated for 18 hr.; the amount of bacterial multiplication was estimated by comparison with standard opacity tubes; the hyaluronidase and spreading activities of the culture fluids were titrated in the usual way. The result of this experiment is recorded in Table 8.

It will be seen that the addition of glucose to the medium results in an eightfold increase in bacterial multiplication and an approximately fivefold increase in hyaluronidase production. The substitution of potassium hyaluronate for glucose does not significantly increase multiplication of the organism, but the concentration of hyaluronidase shows a dramatic ninefold increase as compared with the medium containing glucose; this is accompanied by a considerable increase of spreading activity in the skin. The medium containing 1 unit of hyaluronidase shows a further slight increase of hyaluronidase which may be significant. This experiment, which has been repeated with consistent results, shows that *Cl. welchii* can use a salt of hyaluronic acid as a metabolite and that the inclusion of the specific substrate in the medium induces a greatly enhanced production of hyaluronidase.

Table 8. *Cultivation of* Cl. welchii *in various media*

Medium	Bacterial multiplication. Opacity tube number	Hyaluronidase. Viscosity-reducing units	Dermal diffusion. Highest dilution producing diffusion
3·5% Evans peptone alone	1	13	10^{-5}
3·5% Evans peptone + 0·5% glucose	8	70	10^{-7}
3·5% Evans peptone + 0·5% potassium hyaluronate	8	1340	10^{-9}
3·5% Evans peptone + 0·5% potassium hyaluronate + 1 unit *welchii* hyaluronidase	8	1660	10^{-9}

Infections by *Cl. welchii* are characterized by extremely rapid spread through the tissues and marked generalized toxaemia. The rapid adaptation of this organism in the presence of a salt of hyaluronic acid in the culture medium is of considerable interest in this connexion. It is known that there is a mucinous interfibrillar structure in the tissues, and recently Claude [1940] and Chain & Duthie [1940] have described the extraction from the skin of a substance indistinguishable from hyaluronic acid which is destroyed by hyaluronidase. We have also found that reducing substances are liberated by the action of this enzyme on crude extracts from the skin. Therefore, it appears likely that as soon as *Cl. welchii* start to proliferate in the tissues the hyaluronic acid there is attacked by means of this enzyme. Thus the organism is able to use this metabolite and, at the same time, by destroying the viscosity of this material to increase the permeability of the tissues and render more of the specific substrate accessible. In addition, the presence of hyaluronic acid causes the organism to produce greatly increased amounts of the enzyme. In this way a vicious circle is set up that promotes the rapid extension of the infection.

Further investigations on this adaptive enzyme effect with other strains of *Cl. welchii* are in progress and similar observations on strains of streptococcus and staphylococcus which do and those which do not produce the diffusing factor are being made. An attempt is being made to correlate this phenomenon with the relative virulence or local invasiveness of different strains of these organisms, but results of these observations will be reported in detail elsewhere.

Neither sulphanilamide nor sulphapyridine (M and B 693) inhibit the *in vitro* production of hyaluronidase by *Cl. welchii* when these substances are introduced into the culture medium. Neither of the substances have any direct action on the viscosity of the mucoprotein substrate, nor do they inhibit the *in vitro* activity of the enzyme.

Correlation between hyaluronidase activity and diffusion in the dermis

The close association between hyaluronidase activity and diffusion in the dermis that is obtained with tissue extracts, various bacterial extracts and snake venoms presents a strong *prima facie* case for assuming that the same enzyme is responsible for both phenomena. This assumption is further supported by the isolation of a substance from the skin [Claude, 1940; Chain & Duthie, 1940] which is indistinguishable from hyaluronic acid, and the viscosity of which is reduced by hyaluronidase. We have ourselves observed that enzyme preparations cause the liberation of reducing substances from a crude extract of skin. The fact that diazotized compounds, ascorbic acid and its oxidation products and other artificially prepared substances which reduce the viscosity of the

substrate also diffuse in the skin, though in a slower manner than enzymes, adds support to the belief that both diffusion and viscosity reduction have an identical cause. Recently, however, Madinaveitia *et al.* [1940] have published a warning that it is not justifiable to assay skin-diffusing factors by merely measuring their hyaluronidase activity. Using the method of Bacharach *et al.* [1940], these authors failed to obtain parallel figures for the titrations of diffusing factor potency and hyaluronidase activity measured viscosimetrically and they suggest that it may be possible to separate diffusing factors from hyaluronidase. While the validity and relative accuracy of various methods of assaying diffusing factors is under investigation it may be suggested that conclusions based upon the lack of correspondence between titrations of diffusing factor and of hyaluronidase should be treated with caution. The environmental conditions of the test in the skin and that in the viscosimeter differ profoundly and may exert differential effects upon samples of enzymes from different sources or those from the same source purified by different methods. Viscosimetric tests were carried out by Madinaveitia & Quibell [1940] in 0·66 *M* NaCl and at *p*H 4·6, whereas skin diffusion presumably takes place in isotonic salt at or about neutrality. The marked influence of salt concentration and *p*H on the *in vitro* activity of the enzymes has already been demonstrated; it has also been shown that testicular and bacterial enzymes differ in their responses to changing salt concentration. There is also evidence that reducing substances affect the viscosity of the substrate and the permeability of the tissues; little is known at present about the oxidation-reduction potential of the *in vitro* enzyme system or of the tissues into which these factors are injected. Investigations on this point are in progress. In addition, there are many possible factors concerned in the response of an animal to these substances which it is not possible to control adequately at present. In the existing state of knowledge it is certainly unjustifiable to assay skin-diffusing factors in terms of hyaluronidase activity but, in view of the considerations set out above, it does not follow that the two activities are due to separate agents.

Diffusion in the skin is caused by enzyme preparations that are too weak to be titrated viscosimetrically and those of moderate hyaluronidase content produce significant diffusion at high dilution. The skin test is therefore very much more delicate though much less accurate than viscosimetric assay. In the section describing our methods, we have explained why, for the purposes of this work, we preferred to continue testing for diffusing activity by determining the minimal diffusing dose rather than by the methods devised by Madinaveitia or by Bacharach *et al.* When the limiting diffusing dilution is determined it is not claimed that differences of less than tenfold dilutions can be distinguished, but it is possible to determine whether samples assayed by this method fall into the same order of activity as they do when titrated viscosimetrically. Table 9 contains the result of such a comparison.

It will be seen that, broadly speaking, the enzyme samples fall into the same order of activity by both tests. There is slight overlapping of those samples showing limiting diffusion at dilutions of 10^{-4} and 10^{-5}, but otherwise there is as good agreement as could be expected when the limitations of the animal test are taken into consideration. Further work is required to determine how far the diffusion test of Bacharach *et al.* will give results in agreement with our test, and these comparative observations are in progress. In the meantime the results presented in this table support the belief that diffusion in the skin may be due to hyaluronidase activity. This conclusion is further supported by the work of Chain & Duthie [1940] which has just appeared; these authors find good quanti-

tative agreement between hyaluronidase activity determined viscosimetrically and spreading activity in the skin with various testicular extracts and with leech and bee venoms.

Table 9. *Correlation between diffusion in the dermis and viscosity-reducing activity*

Source of enzyme	Highest dilution producing diffusion in the dermis	Viscosity-reducing units (in 0·146 *M* NaCl)
Cl. welchii filtrate 1*a*	10^{-3}	6·5
Cl. welchii filtrate 1	10^{-4}	13
Cl. welchii (purified by method of Morgan & McClean [1932])	10^{-4}	25
Cl. welchii (purified by method of Robertson *et al.* [1940])	10^{-5}	18
Cl. welchii (purified by method of Morgan & McClean)	10^{-5}	25
Cl. welchii filtrate 2*a*	10^{-5}	29
Testis, Glaxo standard, 1 mg./ml.	10^{-5}	36
Testis (purified by method of Morgan & McClean)	10^{-5}	70
Cl. welchii filtrate 2	10^{-6}	70
Cl. welchii filtrate 3*a*	10^{-7}	920
Cl. welchii filtrate 3	10^{-8}	1340
Cl. welchii filtrate 4	10^{-8}	1660

Summary

1. The association between hyaluronidase activity and diffusion in the tissues of testicular extracts, certain bacterial filtrates and snake venom is described.

2. The influence of *p*H on the hyaluronidase activity of these substances has been investigated.

3. The liberation of reducing substances by the enzymes has been studied. Quantitative comparisons of viscosity-reducing activity and the time relation of both reactions suggest that two separate mechanisms may be involved.

4. The reduction of viscosity and liberation of reducing substances by bacterial enzymes can be completely neutralized by appropriate antisera which also inhibit diffusion in the skin. This neutralization is strictly specific.

5. A study of the influence of the concentration of electrolytes on the reduction of viscosity and liberation of reducing substances by testicular and bacterial enzymes reveals that the relative potency assigned to enzyme samples from different sources is profoundly modified by the salt concentration of the system. Testicular enzymes appear to differ in their behaviour from bacterial enzymes as the salt concentration is reduced.

6. The relation between viscosity and concentration of substrate has been investigated. The viscosity is not proportional to the concentration. The bearing of this observation on estimation of enzyme activity is discussed.

7. There is no correlation between the hyaluronidase activities of enzymes from various sources and their amylase or phosphatase contents, nor with their actions on gastric mucin or several other polysaccharides.

8. The hyaluronidase of Russell's viper venom ('Stypven') is not associated with any action on heparin. Neither this venom nor testicular or bacterial enzymes have any direct action on heparin, nor do they promote the coagulation of heparinized plasma.

9. Diazotized compounds are known to diffuse in the dermis; the observation that they also reduce the viscosity of mucoprotein has been confirmed. These compounds do not liberate reducing substances.

10. Ascorbic acid and its oxidation products cause a rapid fall in the viscosity of mucoprotein without the liberation of reducing substances. They also diffuse in the skin in a manner similar to diazotized compounds.

11. Various reducing agents also cause a fall in viscosity of the substrate and diffuse in the skin. The action of these artificially prepared compounds is compared with that of naturally occurring enzymes. Further investigations on the activity of artificial compounds are in progress.

12. Preliminary experiments on the influence of the inclusion of potassium hyaluronate in the culture medium of *Cl. welchii* are reported. A large increase in the yield of hyaluronidase results. The significance of this increase in relation to the proliferation of these organisms in the tissues is discussed. Further observations with this and other organisms that produce hyaluronidase are in progress.

13. Sulphanilamide and sulphapyridine do not inhibit production of this enzyme by *Cl. welchii* nor do they inhibit the *in vitro* activity of the enzyme.

14. The question whether diffusion in the tissues is due to hyaluronidase is discussed. The correlation between tests for the two activities is considered. Our experimental results support the belief that diffusion in the skin is due to hyaluronidase activity.

We wish to express our appreciation of the interest and advice of Dr B. C. J. G. Knight throughout this investigation.

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18. METABOLISM OF POLYCYCLIC COMPOUNDS

4. PRODUCTION OF A DIHYDROXY-1:2:5:6-DIBENZANTHRACENE FROM 1:2:5:6-DIBENZANTHRACENE

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When anthracene is fed to rats and rabbits different stereoisomeric forms of 1:2-dihydroxy-1:2-dihydroanthracene and their glycuronic acid derivatives are excreted in the urine [Boyland & Levi, 1935; 1936]. Extension of the work to the metabolism of the carcinogenic compound 1:2:5:6-dibenzanthracene in rabbits has led to the isolation of a phenol, dihydroxy-1:2:5:6-dibenzanthracene by Boyland & Levi [1938]. The same compound was later obtained by Dobriner *et al.* [1939]. No positive evidence of the presence of hydrogenated derivatives with asymmetric carbon atoms corresponding to those produced from anthracene has been found, although such compounds might be excreted and later decomposed to give the phenol.

Experimental

Rabbits were fed on the following diet:

Wholemeal flour	35 parts
Wheat germ	30 ,,
Dried milk	10 ,,
Lard containing 0·8% of 1:2:5:6-dibenzanthracene	20 ,,
Cod liver oil	2 ,,
NaCl	2 ,,
Dried yeast	1 part

The rabbits which were maintained on this diet for many months died generally with cirrhosis of the liver, similar to that described as resulting from injection of 1:2:5:6-dibenzanthracene into rabbits [Polson, 1936; Claude, 1937; Burrows & Boyland, 1938]. In some experiments in which the same 1:2:5:6-dibenzanthracene diet was fed to rats no cirrhosis was produced but the animals died in 6–10 weeks. With both species proteinuria frequently developed during the experiments.

The animals were kept in metabolism cages and the urine was collected daily, filtered through kieselguhr and stored at 0–5°. The urine was fluorescent and the isolation of products was followed by continuous examination of extracts in ultraviolet light.

Examination of sulphur compounds

The partition of the sulphur present in the urine was determined as described by Folin [1905] and the results obtained with two rabbits to which 1:2:5:6-dibenzanthracene was administered are given in Table 1. The significance of the

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results was calculated by the method of '*t*' [Fisher, 1936]. In both cases there is an increase in the proportion of ethereal sulphate present, indicating that the dibenzanthracene may be partly excreted as a sulphuric ester of a hydroxy derivative.

In one case (rabbit B) there was a considerable increase in the amount of neutral sulphur particularly after the rabbit had been on the dibenzanthracene

Table 1. *The effect of administration of 1:2:5:6-dibenzanthracene to rabbits on the sulphur partition in the urine*

Rabbit A. 7 days on normal diet, then on diet containing 1:2:5:6-dibenzanthracene

Day	Percentage of total sulphur		
	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	51·8	6·2	42·0
2	49·5	5·5	45·0
3	44·7	7·0	48·3
6	41·9	2·6	55·5
7	42·8	3·4	53·8
13	63·5	7·8	29·7
14	50·5	3·2	46·3
15	44·5	9·1	46·4
27	53·0	11·0	36·0
35	53·8	13·2	33·0
57	34·7	14·6	50·7
63	50·7	7·6	41·7
78	44·1	3·8	52·1
Mean on normal diet		4·9	48·9
Mean on dibenzanthracene diet		8·8	42·1
Probability '*P*' that difference in means is real		0·05	0·2

Rabbit B. 40 days on normal diet, then on diet containing 1:2:5:6-dibenzanthracene

Day	Percentage of total sulphur		
	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	71·8	4·8	23·4
4	74·7	5·6	19·7
8	68·3	11·8	19·9
11	69·9	5·6	24·5
16	79·6	6·0	14·4
40	69·5	7·5	23·0
42	60·1	10·2	29·7
46	66·5	5·8	27·7
47	5·8	11·2	30·4
49	74·5	4·5	21·0
53	60·5	6·2	33·3
103	28·0	8·0	64·0
109	31·6	8·5	54·5
118	37·7	8·1	54·2
		6·9	20·6
		8·5	37·2
		0·2	0·02

Table 2. *The effect of administration of 3:4:5:6-dibenzcarbazole to rabbits on the sulphur partition in the urine*

Rabbit C. 13 days on normal diet, then on diet containing 0·16% 3:4:5:6-dibenzcarbazole

Day	Percentage of total sulphur		
	Inorganic sulphate	Ethereal sulphate	Neutral sulphur
1	58·0	4·7	37·3
2	53·7	6·6	39·3
6	47·4	5·8	46·8
7	40·4	6·1	53·5
13	53·1	7·8	39·1
14	34·9	12·8	52·3
15	19·8	12·8	67·4
20	46·7	11·6	41·7
26	28·4	12·1	59·5
33	30·9	17·7	51·4
42	47·1	8·7	44·2
61	31·4	26·8	41·8
74	32·1	12·9	55·0
Mean on normal diet		6·2	43·2
Mean on dibenzcarbazole diet		12·8	51·7
Probability '*P*' that increase is real		0·05	0·05

diet for 50 days. It seems probable that this increase in neutral sulphur is due to proteinuria following hepatic and renal injury. No mercapturic acid was isolated whereas if the increased neutral sulphur had been due to dibenzanthracene mercapturic acid, large amounts of this compound would have been present and its isolation should not have presented difficulties.

Some similar results with the carcinogenic 3:4:5:6-dibenzcarbazole are given in Table 2. Again there is a marked increase in the amount of ethereal sulphate and a small but definite increase in the neutral sulphur fraction. Other chemical investigations indicated that 3:4:5:6-dibenzcarbazole is excreted as a fluorescent hydroxy derivative. It is not possible to say whether in this case the increase in neutral sulphur is due to mercapturic acid or excreted protein, but rabbits to which 3:4:5:6-dibenzcarbazole was administered developed proteinuria.

Other determinations were made on the collected urine of 12 rats on normal diets and diets containing carcinogenic compounds. On adding 1:2:5:6-dibenzanthracene to the diet the ethereal sulphate rose slightly, from 8·1 to 9·6 %, and the neutral sulphur from 24·1 to 26·7 % of the total sulphur in the urine. The addition of 3:4:5:6-dibenzcarbazole to the rats' diet similarly increased the ethereal sulphate from 11·3 to 14·7 % and the neutral sulphur from 31·6 to 34·6 % of the total urine sulphur. These experiments indicate that these two carcinogenic substances are also excreted as sulphuric esters in rat's urine.

Isolation of dihydroxy-1:2:5:6-dibenzanthracene

This substance was obtained from the urine in two ways:

(*a*) The dried and powdered kieselguhr and precipitate were extracted in a Soxhlet apparatus with ether for some hours. The fluorescent ether extract was washed repeatedly with very dilute Na_2CO_3 solution until no further acidic material was present. This process removed much acid, the main part of which appeared to consist of stearic acid; M.P. after two crystallizations from aqueous alcohol 60–61°, equiv. wt. 280, 288 (stearic acid equivalent weight 284, M.P. 62·2°).

The residual ether was then extracted two or three times with dilute NaOH. The acidified extract gave a yellow flocculent precipitate of a phenol, which was collected and dried and recrystallized from acetic acid.

(*b*) The filtered urine in 2 l. lots was acidified, shaken with butyl alcohol in a large funnel, and allowed to stand for 12 hr. The emulsion remaining in the butyl alcohol layer was largely removed by filtration through kieselguhr. The butyl alcohol was then mixed with one volume of light petroleum, washed with water, and extracted with several small portions of dilute NaOH until the extracts were only faintly coloured. The combined alkaline extracts were washed with ether to remove dissolved butyl alcohol, first made acid with HCl, then alkaline with Na_2CO_3, and the phenolic material extracted with ether. It was re-extracted with NaOH solution, precipitated with HCl, filtered, dried and recrystallized from acetic acid. It was identical with that obtained from the kieselguhr. From the two sources about 10 mg. were isolated from each litre of urine.

Dihydroxy-1:2:5:6-dibenzanthracene crystallized in yellow needles which darken on exposure to light; M.P. 340–350° with decomposition; soluble in alcohol, acetone and ether to give violet-fluorescent solutions; almost insoluble in $CHCl_3$ and benzene. It was not extracted from ethereal solution by Na_2CO_3 solution. The solution in NaOH was yellow with a bright green fluorescence and

was fairly stable in air. The aqueous and alcoholic solutions gave no reaction with $FeCl_3$. A 1 % solution of the substance in dioxane ($c=1$ %) showed no optical activity. Analysis (Schoeller), found: C, 84·2 %; H, 5·6 %. $C_{22}H_{14}O_2$ requires: C, 85·2 %; H, 5·5 %.

This phenol could be obtained by alkaline hydrolysis of the purified diacetyl derivative and was more conveniently purified and obtained in better yield in this way.

Diacetoxy-1:2:5:6-dibenzanthracene. The dihydroxy-1:2:5:6-dibenzanthracene obtained from urine was dissolved in one volume of pyridine and the solution boiled with ten volumes acetic anhydride for 5 min. The solution was hydrolysed with water and the product crystallized from acetic acid in colourless plates, M.P. 291°. It was slightly soluble in ethyl alcohol and the solution had a blue-violet fluorescence.

Analysis (Weiler), found: C, 78·0 %; H, 4·5 %. $C_{26}H_{18}O_4$ requires: C, 78·1 %; H, 4·6 %.

Diacetoxy-1:2:5:6-dibenzanthraquinone. Diacetoxy-1:2:5:6-dibenzanthracene on warming with CrO_3 in acetic acid gave a quinone which crystallized from hot acetic acid in yellow needles, M.P. 294–296°. It was soluble in alcohol and dissolved slowly in alkaline $Na_2S_2O_4$ to give a clear orange solution. 1:2:5:6-Dibenz-9:10-anthraquinone gives a red solution in alkaline $Na_2S_2O_4$. Analysis (Weiler), found: C, 78·3 %; H, 4·0 %. $C_{20}H_{16}O_6$ requires: C, 78·6 %; H, 3·8 %.

Dimethoxy-1:2:5:6-dibenzanthracene. Dihydroxy-1:2:5:6-dibenzanthracene treated with dimethyl sulphate in NaOH solution gave a methyl ether which crystallized from hot acetic acid in pale brown needles, M.P. 244–245°. It gave violet-fluorescent solutions in acetone and alcohol. Analysis (Schoeller), found: C, 85·0 %; H, 5·7 %. $C_{24}H_{18}O_2$ requires: C, 85·3 %; H, 5·3 %.

Dimethoxy-1:2:5:6-dibenzanthraquinone. Oxidation of the dimethyl ether with CrO_3 in acetic acid gave poor yields of the quinone, which crystallized from ethyl alcohol in red plates, M.P. 264°. Analysis (Schoeller), found: C, 77·2 %; H, 4·40 %. $C_{24}H_{16}O_4$ requires: C, 78·0 %; H, 4·32 %. The solution in NaOH with $Na_2S_2O_4$ was clear yellow.

Dihydroxy-1:2:5:6-dibenzanthraquinone. The diacetyl derivative of the quinone when warmed with 2*N* NaOH gave a deep blue solution from which a nearly black Na salt separated on standing. The blue solution turned orange on being warmed with Zn dust or $Na_2S_2O_4$.

The hydroxyquinone was prepared by warming the diacetyl derivative with alcoholic alkali for a few minutes. The deep blue solution was poured into dilute acid, when a red gelatinous precipitate separated. This was collected, dried and recrystallized from glacial acetic acid, when it formed deep red needles, M.P. 350°. Analysis (Schoeller), found: C, 77·0 %; H, 3·8 %. $C_{22}H_{12}O_4$ requires: C, 77·7 %; H, 3·5 %.

On heating with boracetic anhydride in acetic anhydride the dihydroxyquinone did not form a boroacetate, but the original quinone acetate was recovered. The substance was readily methylated with dimethyl sulphate in NaOH. Its blue alkaline solution was changed to green by the addition of diazotized sulphanilic acid.

The quinone (100 mg.) was oxidized with acid $KMnO_4$ under conditions identical with those described for the oxidation of 1:2:5:6-dibenzanthraquinone to anthraquinone tetracarboxylic acid [Cook, 1931]. No anthraquinone tetracarboxylic acid could be isolated, whereas had the hydroxyl groups been situated in the side rings this product should have been obtained.

Absorption spectra

The absorption spectra were measured by means of a Hilger medium quartz spectrograph and a Spekker photometer [cf. Mayneord & Roe, 1935]. Alcohol was used as solvent throughout. 1:5-Dihydroxyanthraquinone was sparingly soluble in alcohol and gave varying results when the suspension was warmed to hasten solution. The solution finally used was obtained by shaking for some hours in the dark.

A comparison of the spectrum of the phenol (dihydroxy-1:2:5:6-dibenzanthracene, Fig. 1) with that of 1:2:5:6-dibenzanthracene [Mayneord & Roe, 1935] indicates clearly that it is a 1:2:5:6-dibenzanthracene derivative. The spectrum is shifted 50–100 Å. towards the visible compared with 1:2:5:6-dibenzanthracene; its absorption intensity is diminished, and its long-wave band system is simpler in form.

Substitution by a methyl group in a side-ring of 1:2:5:6-dibenzanthracene, e.g. 2′- or 3′-methyl-1:2:5:6-dibenzanthracene, has very little effect on the absorption spectrum. The resulting spectrum shows slightly lower band-intensities and 2–20 Å. band-shifts towards the visible compared with that of 1:2:5:6-dibenzanthracene [E. Roe, unpublished results]. No data are available on the effect of the substitution by hydroxyl groups in the side-rings, and although the spectrographic evidence below suggests that in the phenol the hydroxyl groups are substituted in the anthracene nucleus, it does not eliminate the possibility of substitution in the side-rings.

In order to obtain further information as to the position of the hydroxyl groups in the phenol, the absorption spectrum of its quinone has been examined and compared with that of 1:2:5:6-dibenzanthraquinone (Fig. 1). The main absorption band of the latter at 2962 Å. is shifted to 3042 Å. in the dihydroxy-derivative and its 2375 Å. band is similarly shifted to 2484 Å. There are greater differences in the long-wave region however, the band at 3900 Å. in 1:2:5:6-dibenzanthraquinone being replaced by one at 4535 Å. in its dihydroxy-derivative.

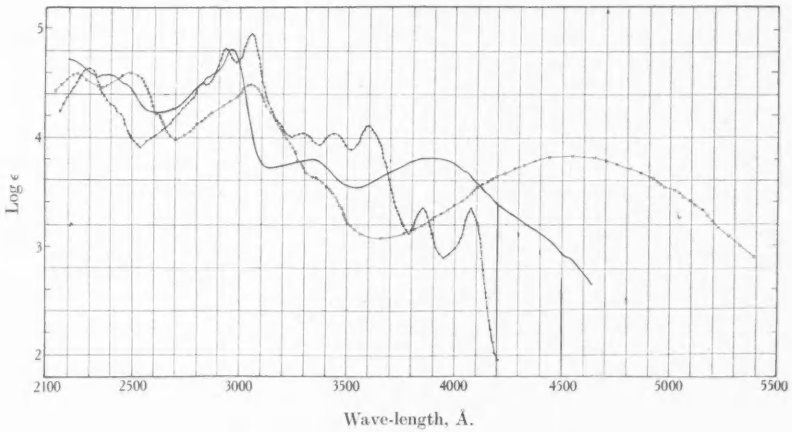


Fig. 1. •—• Dihydroxy-1:2:5:6-dibenzanthracene.
o—o Dihydroxy-1:2:5:6-dibenzanthraquinone. —— 1:2:5:6-Dibenzanthraquinone.

These spectra may be compared with those of anthraquinone and its 2:6- and 1:5-dihydroxy-derivatives (Fig. 2). The spectrum of 2:6-dihydroxyanthraquinone is similar in form to that of anthraquinone and is shifted 200–300 Å. towards the visible. 1:5-Dihydroxyanthraquinone presents a different type of spectrum. The short-wave bands are shifted slightly towards the visible compared with anthraquinone, but the band at 3230 Å. in the latter is replaced by a band at 4230 Å. in the 1:5-dihydroxy-compound, a difference of 1000 Å.

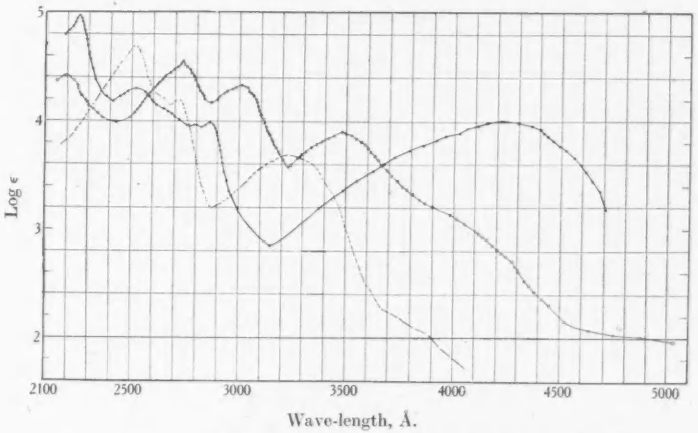


Fig. 2. ——— Anthraquinone. •—• 1:5-Dihydroxyanthraquinone. o—o 2:6-Dihydroxyanthraquinone.

Table 3. *Wave-lengths of maxima, with extinction coefficients*

λ = wave-length in Å.; $\epsilon = \log \frac{I_0}{I} \Big/ l.c$

c = concentration in g.-mol. per l. l = cm.

λ	log ϵ	λ	log ϵ
Dihydroxy-1:2:5:6-dibenzanthracene		9:10-Anthraquinone	
2298	4·64	2514	4·69
2823	4·49	2711	4·19
2928	4·81	3230	3·69
3045	4·95		
3289	4·04	2:6-Dihydroxy-9:10-anthraquinone	
3430	4·04		
3590	4·12	2190	4·41
3848	3·34	2733	4·54
4070	3·35	3004	4·33
		3475	3·90
Dihydroxy-1:2:5:6-dibenz-9:10-anthraquinone		1:5-Dihydroxy-9:10-anthraquinone	
2240	4·59	2253	4·97
2484	4·59	2515	4·30
3042	4·49	2768	4·00
4535	3·83	2860	3·99
1:2:5:6-Dibenz-9:10-anthraquinone		4230	4·00
2375	4·58		
2962	4·81		
3328	3·79		
3900	3·81		

This change is similar to that occurring in the spectrum of the dihydroxy-1:2:5:6-dibenzanthraquinone derived from the phenol when compared with its parent 1:2:5:6-dibenzanthraquinone and it suggests that the hydroxyl groups in the phenol may occupy the 1:5-positions of the anthracene nucleus, i.e. that the phenol isolated from rabbit urine is 4:8-dihydroxy-1:2:5:6-dibenzanthracene.

Tests for carcinogenic activity

The dihydroxy-1:2:5:6-dibenzanthracene was not sufficiently soluble in benzene to be applied by painting to mouse skin in that solvent. Experiments were made with an acetone solution of the substance (0·1 %), with which the backs of 10 mice were painted twice weekly. None of the mice developed tumours although one mouse survived the treatment for 16 months and another for $18\frac{1}{2}$ months. In another experiment 10 mice were injected with 2 doses each of 1 mg. of the phenol suspended in olive oil, with an interval of one week between injections. All the mice died without tumours between the 51st and 119th day following the injection. This is in agreement with the findings of Dobriner *et al.* [1939].

Discussion

It has unfortunately been necessary to interrupt this work before the suggested structure of the dihydroxydibenzanthracene could be tested by synthesis and also before making a complete examination of the excretion of dibenzanthracene in the rat.

Rats and rabbits produce different products from anthracene [Boyland & Levi, 1935; 1936] and also react differently to 1:2:5:6-dibenzanthracene. The injection of 1:2:5:6-dibenzanthracene into rats readily produces tumours at the site of injection while similar injections into rabbits do not [Burrows & Boyland, 1938]. It is possible that this difference in response is due to a difference in the metabolism of the carcinogen analogous to that found in anthracene metabolism.

The non-carcinogenic phenol isolated from the urine of rabbits which were fed with the carcinogenic 1:2:5:6-dibenzanthracene may have been present as a tetrahydroxytetrahydro-1:2:5:6-dibenzanthracene or the corresponding glycuronide if the metabolism of dibenzanthracene is similar to that of anthracene. In that case the dibenzanthracene derivative must be less stable than the corresponding dihydroxydihydroanthracene which was converted into 1-hydroxyanthracene on gentle heating with dilute acid.

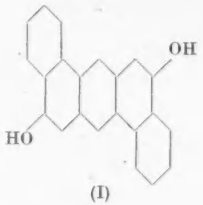
The positions of the hydroxyl groups in the molecule of the phenol cannot definitely be decided. The dihydroxydibenzanthracene even in alkaline solution was stable to air and did not give colour reactions with ferric chloride. It is therefore improbable that the hydroxyl groups are attached to adjacent carbon atoms or to carbon atoms in the *p*-positions in a benzene ring.

The fact that oxidation with $KMnO_4$ did not yield anthraquinone tetracarboxylic acid indicates that the hydroxyl groups are not in the side rings of the dibenzanthracene molecule. Cason & Fieser [1940] have synthesized 4′:8′-dihydroxy-1:2:5:6-dibenzanthracene which they describe as being probably identical with the substance excreted when dibenzanthracene is injected into rats and mice. This differs from the product excreted by rabbits which Cason & Fieser [1940] tentatively suggest is 3′:7′-dihydroxy-1:2:5:6-dibenzanthracene.

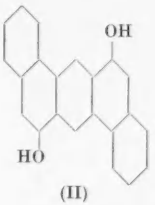
The formation of the diacetoxy-, dimethoxy- and dihydroxy-dibenzanthraquinone excludes the 9 and 10 positions. The non-formation of a boracetic ether

suggests that neither of the hydroxyl groups is in the *peri* position to the quinone groups. The ease with which the dihydroxyquinone reacts with dimethyl sulphate is also in agreement with the hypothesis that the hydroxyl groups are in 3:7 positions. On the other hand the spectrographic evidence suggests that the compound might be 4:8-dihydroxy-1:2:5:6-dibenzanthracene.

Possible formulae for the excretion product therefore include 3:7-dihydroxy-1:2:5:6-dibenzanthracene (I), 4:8-dihydroxy-1:2:5:6-dibenzanthracene (II) and 3:8-dihydroxy-1:2:5:6-dibenzanthracene (III).



(I)



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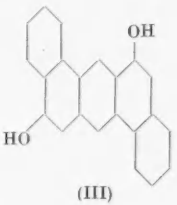

(III)

SUMMARY

1. The administration of 1:2:5:6-dibenzanthracene or 3:4:5:6-dibenzcarbazole to rats or rabbits causes an increase in the excretion of ethereal sulphate in the urine.

2. A phenolic derivative, dihydroxy-1:2:5:6-dibenzanthracene was isolated from the urine of rabbits fed on a diet containing 1:2:5:6-dibenzanthracene. This phenol has been characterized and several derivatives prepared.

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19. VITAMIN B_1 AND BACTERIAL OXIDATIONS

1. DEPENDENCE OF ACETIC ACID OXIDATION ON VITAMIN B_1

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Vitamin B_1 is known to be a necessary factor for the growth of certain bacteria [Knight, 1937, 1, 2; cf. also Tatum *et al.* 1936; Barron & Lyman, 1939; Nilsson *et al.* 1938]. Such bacteria can be grown on media containing extremely minute quantities of the vitamin, and may even be trained to grow in the absence of the vitamin [Wood *et al.* 1938], in which case the organisms apparently may acquire the ability to synthesize it [Silverman & Werkman, 1939, 2]. The metabolism of bacteria grown on vitamin B_1-deficient media may be greatly enhanced by the addition of small quantities of the vitamin to suspensions of the organism. This has been shown by Hills [1938] in the case of *Staphylococcus aureus*, and by Silverman & Werkman [1938] in the case of propionic acid bacteria. The former author reported a marked stimulation of the oxidation of pyruvate and of the anaerobic breakdown of pyruvate by *Staphylococcus* on the addition of the vitamin. Silverman & Werkman [1938; 1939, 3] found that the rate of anaerobic breakdown of pyruvic acid by propionic acid bacteria and by *Lactobacillus mannitopoeus* is increased on the addition of the vitamin to washed suspensions of the organisms.

We have carried out a systematic study of the effects of vitamin B_1 on the oxidative powers of propionic acid bacteria grown on vitamin B_1-deficient media and of the various factors which influence the action of the vitamin. It is proposed to deal in this paper with the effects of the vitamin on various oxidations stimulated by these bacteria. We have already reported that the vitamin appears to be essential for the oxidation of acetic acid by these organisms [Quastel & Webley, 1939].

Preparation of vitamin B_1-deficient propionic acid bacteria

We have used a strain of propionic acid bacteria obtained from the National Collection of Type Cultures (Ref. No. 4759). The organism is grown on Petri dishes containing a medium deficient in vitamin B_1. This medium is prepared as follows. A mixture of 20 g. 'Difco' peptone, 6 g. NaCl and 40 g. agar-agar is dissolved in 1 l. of distilled water, the mixture being warmed until solution has taken place. *N* NaOH solution is now added until the *p*H is 9·0, using bromothymol blue as indicator, and the mixture is autoclaved at 125° for 1 hr. The medium is finally filtered through absorbent cotton wool and the *p*H adjusted to 6·6. This medium may be autoclaved many times without its consistency, after cooling in Petri dishes, being appreciably affected. Occasionally we have found it necessary to autoclave the medium a second time at *p*H 9·0 to reduce the vitamin B_1 content to the low levels required for the preparation of the vitamin-deficient bacteria.

The plates are spread with an 18 hr. culture of the propionic acid bacteria which have been previously grown on the vitamin-deficient medium. The organism is grown aerobically at 37° for 18 hr., after which it is washed off the plates with sterile 0·16 *M* saline. The suspension of organisms is washed twice with saline by centrifuging. It is finally suspended in saline and stored at 0°. 1 ml. of the suspension is usually made to contain about 10 mg. dry weight of the bacteria.

Measurement of the respiration of the bacteria

This is accomplished with the use of the Barcroft, and Warburg, respiratory apparatus. 1 ml. of the bacterial suspension is usually employed and to this are added solutions of phosphate buffer, substrate etc., to produce a final volume of 3 ml. The measurements of O_2 uptake are carried out at 37° at *p*H 7·4 in an atmosphere of air. The experiments are generally run for 1 hr.

Measurement of the weight of bacteria

This is carried out by means of the Spekker photoelectric absorptiometer. The opalescence of a known weight of the bacteria suspended in saline is measured on the instrument, using a standard light filter, and a calibration curve is drawn relating opalescence to bacterial weight. The curve is linear for densities of the organism not exceeding 0·25 mg. dry weight per ml. With the aid of this curve it is a simple matter at the termination of each experiment to dilute the contents of the respiration flask until a suitable density of the organism is obtained, to determine the opalescence on the absorptiometer and to estimate from the curve the weight of bacteria present in the suspension.

The effect of vitamin B_1 on the oxidation of acetate by vitamin B_1-deficient propionic acid bacteria

Acetic acid is known from the work of Stone *et al.* [1936] to be a hydrogen donator in presence of propionic acid bacteria. Little is known of the metabolism of acetic acid in presence of these bacteria, but Wood *et al.* [1937] conclude that their evidence, derived from analyses of the products of glucose fermentation, points to the conversion of acetic acid into succinic acid.

The addition of acetate to suspensions of vitamin B_1-deficient propionic acid bacteria increases the rate of O_2 uptake. The further addition of vitamin B_1 results in a large acceleration of the rate of oxidation of acetate. A typical result is shown in Table 1. The rate of O_2 uptake by the bacteria in the presence of acetate and vitamin B_1 is linear. As the oxidation proceeds, the *p*H of the solution in the respiration flask steadily increases, sodium bicarbonate and carbonate being formed. The vitamin has but little effect on the respiration of the bacteria in the absence of added substrates.

Table 1

Each manometer vessel contained 1·0 ml. suspension of freshly washed and freshly grown *B. acidi propioni*, 0·5 ml. *M*/5 sodium phosphate buffer *p*H 7·4, the amounts of substrates and vitamin B_1 stated below and 0·16 *M* saline to bring the volume to 3 ml. O_2 uptakes were determined in an atmosphere of air at 37° for a period of 2 hr.

Substrate added	Vitamin B_1 added μg.	O_2 uptake in 2 hr. μl.
Nil	Nil	197
0·3 ml. *M*/10 Na acetate	Nil	563
Nil	0·25	245
0·3 ml. *M*/10 Na acetate	0·25	1202

Variation of the velocity of O_2 uptake by propionic acid bacteria in presence of acetate with the concentration of vitamin B_1

The velocity of respiration of the vitamin B_1-deficient bacteria in presence of 0·01 *M* sodium acetate rises sharply as the concentration of vitamin B_1 present is increased. A maximum is reached at a concentration of approximately 30 μg. vitamin B_1 per 100 ml., i.e. 7×10^{-7} *M*. The velocity of O_2 uptake in presence of acetate and in absence of added vitamin B_1 is variable, depending on the conditions of culture and age of the organism. Usually the addition of acetate to a suspension of vitamin B_1-deficient bacteria increases the rate of O_2 uptake, the increase being most probably determined by the small quantities of the vitamin still present in the organism. The variation of the rate of O_2 uptake of the vitamin-deficient bacteria in presence of 0·01 *M* sodium acetate with variation of the amounts of vitamin B_1 added to the suspension of cells is shown in Fig. 1. It

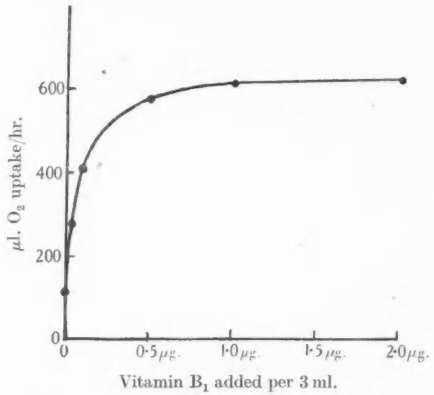

Fig. 1. O_2 uptake by propionic acid bacteria in presence of 0·01 *M* Na acetate and varying quantities of vitamin B_1.

will be noted that a large increase in the rate of respiration in the presence of acetate is secured by the addition of only 0·1 μg. vitamin B_1 to the 3 ml. of cell suspension in the respiration flask.

If propionic acid bacteria are grown on a peptone-agar medium to which vitamin B_1 has been added, suspensions of these bacteria oxidize acetate at a much greater rate than holds when the bacteria are grown on vitamin B_1-free medium. Moreover, the addition of the vitamin to suspensions of bacteria grown on a vitamin B_1-rich medium does not increase the rate of oxidation of acetate.

O_2 uptake and bacterial count

The increased O_2 uptake secured by the addition of vitamin B_1 to suspensions of vitamin B_1-deficient bacteria in presence of acetate is not due to multiplication of the organisms in the respiration flask under our experimental conditions. This is shown by adding the same number of cells to a phosphate-acetate medium, in the absence and in the presence of vitamin B_1, measuring the O_2 uptakes at 37° for 1 hr. and making bacterial counts at the end of this period using a blood-counting chamber. A representative result is shown in Table 2.

Table 2

Conditions as in Table 1

Substance added to 1 ml. bacterial suspension	μl. O_2 consumed	No. of bacterial cells present at end of experiment
Nil	120·9	$10{\cdot}6 \times 10^9$
0·25 μg. vitamin B_1	152·6	$9{\cdot}4 \times 10^9$
0·01 *M* Na acetate	380·0	$9{\cdot}8 \times 10^9$
0·01 *M* Na acetate + 0·25 μg. vitamin B_1	821·3	$9{\cdot}5 \times 10^9$

Oxidation of acetic acid by propionic acid bacteria

Analyses were made of the acetic acid remaining in the respiration flask after the consumption of O_2 by the bacterial suspension, and the amount of acetic acid which had disappeared was compared with the amount of O_2 taken up.

Acetic acid was estimated in the following manner. A mixture of 3 g. anhydrous Na_2SO_4 and 1·5 ml. H_2SO_4 (2 parts conc. H_2SO_4 to 1 of water) was placed in a small pyrex flask and to this were added 2 ml. of the fluid in the respiration flask after the bacteria had been removed by centrifuging. The volume was made up to 6 ml. with distilled water and the flask was connected to a worm condenser and then to a Liebig condenser, ground glass joints being used throughout. The worm condenser and its outlet to the Liebig condenser were swathed with cotton wool. The end of the Liebig condenser dipped into 30 ml. of distilled water contained in a receiving flask. Distillation was carried out for 20 min. using a microburner under the distillation flask. The worm condenser prevented droplets of the fluid in the distillation flask from passing into the receiving vessel, but condensation of the steam containing the acetic acid took place only in the Liebig condenser. The microburner was removed after 20 min. distillation, the apparatus was disconnected, 5 ml. water were added to the distillation flask via the worm condenser and the apparatus was reconnected. Distillation was carried out for a further 20 min., after which the microburner was finally removed, and 30 ml. water were poured down the Liebig condenser (for washing purposes) into the receiving flask. The volume of fluid in this flask was finally 70 ml. The acetic acid present was titrated with CO_2-free N/100 NaOH, using a constant quantity of bromothymol blue as indicator. The method gave accurate and consistent results as long as the volumes mentioned above were adhered to.

Table 3

Substance added to the bacterial suspension	μl. O_2 consumed in 2 hr.	μl. O_2 consumed due to the acetate	μl. acetic acid utilized	Ratio $\frac{O_2 \text{ consumed}}{\text{Acetic acid utilized}}$
Exp. 1				
Nil	197	—	—	—
0·25 μg. vitamin B_1	245	—	—	—
0·01 *M* acetate	563	366	193	1·90
0·01 *M* acetate + 0·25 μg. vitamin B_1	1202	957	486	1·97
Exp. 2				
Nil	195	—	—	—
20 μg. cocarboxylase	221	—	—	—
0·01 *M* acetate	446	251	132	1·90
0·01 *M* acetate + 20 μg. cocarboxylase	1397	1176	591	1·99

Typical results giving the ratio of O_2 consumed by the bacteria to the acetic acid utilized are given in Table 3. The conditions were those quoted in Table 1. The acetic acid utilized by the bacteria is expressed in terms of gas (i.e. 1 g. mol. $\equiv$ 22·4 l. gas).

The results show that the ratio of O_2 consumed to acetic acid utilized, whether vitamin B_1 has been added to the suspension of vitamin-deficient propionic acid bacteria or not, is approximately 2·0.

This value is to be expected if complete oxidation of acetic acid occurs according to the following equation:

$$CH_3COOH + 2O_2 = 2CO_2 + 2H_2O.$$

Effects of vitamin B_1 on the oxidation of fatty acids by vitamin B_1-deficient propionic acid bacteria

The lower fatty acids, formic, propionic and butyric, are oxidized by propionic acid bacteria, but the addition of vitamin B_1 has a much less marked effect on the oxidation of these acids than it has upon that of acetic acid. Representative results are shown in Table 4. It will be seen that whereas the rates of respiration

Table 4

O_2 uptakes of vitamin B_1-deficient propionic acid bacteria in the presence of the Na salts of fatty acids and in the presence and absence of vitamin B_1. Each vessel contained 1 ml. freshly washed suspension of the bacteria, 0·5 ml. *M*/5 Na phosphate buffer *p*H 7·4, 0·1 ml. of a solution containing 0·01 *M* $MgCl_2$ and 0·024 *M* KCl, 0·3 ml. *M*/10 solution of the Na salt of the fatty acid, 0·2 ml. of the solution of vitamin B_1 and 0·9 ml. saline. Respiration measured in air at 37° $Q_{O_2} = \mu$l. O_2 uptake per hour per mg. dry wt. of bacteria.

Substrate	Q_{O_2} In absence of added vitamin B_1	Q_{O_2} In presence of 4 μg. added vitamin B_1
Nil	10·5	12·4
0·01 *M* Na formate	22·5	24·9
0·01 *M* Na acetate	23·4	65·3
0·01 *M* Na propionate	20·7	37·8
0·001 *M* Na butyrate	19·0	25·1

in the presence of the fatty acids and in absence of added vitamin B_1 are approximately the same, the rates differ most markedly in the presence of the vitamin. The addition of the vitamin has but little effect on the oxidation of formate and butyrate, but is definite with propionate and most marked with acetate. The conditions of the experiments, typical results of which are shown in Table 4, are different from those mentioned earlier in that a mixture of magnesium ions and potassium ions was added to the solution in the respiration flask. The reasons for the addition of these ions will be given in a later paper.

It is of interest to note that Stone *et al.* [1936] have shown that resting propionic acid bacteria activate acetic and propionic acids to reduce methylene blue under anaerobic conditions, whilst relatively little or no activation takes place with formic and butyric acids.

Is acetate oxidized via pyruvate in presence of propionic acid bacteria?

It follows from the results quoted in Table 4 that not all oxidations brought about by vitamin B_1-deficient propionic acid bacteria are materially affected by the addition of vitamin B_1. This brings up the question as to whether the accelerating action of vitamin B_1 is primarily due to its effect upon the oxidation

of an intermediate substance common to the breakdown products of acetic and propionic acids but not to those of formic and butyric acids. The obvious possible intermediate is pyruvic acid, the rate of oxidation of which by vitamin B_1-deficient propionic acid bacteria was found to be much increased by the addition of vitamin B_1, as is seen in Table 5.

Table 5

Conditions as in Table 4

Substrates added to bacterial suspension	Q_{O_2}
Nil	12·2
4 μg. vitamin B_1	14·5
0·01 *M* Na pyruvate	24·4
0·01 *M* Na pyruvate + 4 μg. vitamin B_1	42·8

The fact that propionic acid bacteria readily convert lactic acid into propionic acid makes it likely that the reverse process can take place, as pointed out by Stone *et al.* [1936]. It may be shown, with the use of vitamin B_1-deficient bacteria, that oxidation of propionic acid leads to the accumulation of a ketonic acid. When vitamin B_1 is added to the system the amount of ketonic acid accumulating is much diminished. Although the ketonic acid has not been isolated, the fact that it disappears in the presence of vitamin B_1 makes it most probable that the ketonic acid in question is pyruvic acid. This is shown much more clearly when glucose is used as the substrate for oxidation in presence of the vitamin-deficient bacteria. Here there is a rapid formation of a ketonic acid, the accumulation of which is greatly diminished by the presence of vitamin B_1. Its identity with pyruvic acid is made reasonably certain by the fact that Wood *et al.* [1937] have isolated this ketonic acid from the products of breakdown of glucose by the propionic acid bacteria. No other aldehydes or ketones were detected by these authors. The same phenomenon may be shown when lactate or glycerol is used as substrate for oxidation with the vitamin-deficient bacteria.

It is of some interest that the yield of pyruvic acid from lactic acid, when this is oxidized by the propionic acid bacteria, is much less than that from glucose. This shows that the oxidation of glucose does not depend on preliminary fission into lactic acid, and lends support to the view expressed by Wood *et al.* [1937] that the metabolism of glucose by the propionic acid bacteria follows the paths which are followed in muscle and yeast.

Representative results showing the rates of accumulation of pyruvic acid in the absence and presence of vitamin B_1 are given in Table 6. The yield of pyruvic

Table 6

Conditions as in Table 1

Substrates added to bacterial suspension	Vitamin B_1 added μg.	Dry wt. of bacteria mg.	Pyruvic acid liberated in 1 hr. μl.
Nil	Nil	10·5	Nil
Nil	4	10·5	Nil
0·01 *M* glucose	Nil	11·7	139·8
0·01 *M* glucose	4	11·7	86·0
0·01 *M* Na lactate	Nil	7·5	40·3
0·01 *M* Na lactate	4	7·5	6·7
0·01 *M* glycerol	Nil	10·3	26·9
0·01 *M* glycerol	4	10·3	10·0
0·01 *M* Na propionate	Nil	11·7	10·0
0·01 *M* Na propionate	4	11·7	Nil
0·01 *M* Na acetate	Nil	10·5	Nil
0·01 *M* Na acetate	4	10·5	Nil

acid is expressed in terms of μl. gas. It was estimated in the following manner. The fluid in the respiration flask at the end of the experiment was centrifuged. To 2 ml. of the supernatant fluid was added 1 ml. dinitrophenylhydrazine reagent (0·7 g. 2:4-dinitrophenylhydrazine hydrochloride dissolved in 250 ml. *N* HCl). This mixture was allowed to stand for 10 min. at room temperature and then 2 ml. *N* NaOH were added to it. After allowing it to stand further for a few minutes with a little shaking, 5 ml. water were added. The solution was compared colorimetrically with solutions prepared in a similar way from known quantities of pyruvic acid (see Jowett & Quastel [1937]). The Spekker photoelectric absorptiometer was used for the colorimetric comparisons.

It will be observed from the results quoted in Table 6 that pyruvic acid accumulates during the oxidation of propionic acid by the vitamin-deficient bacteria in the absence of added vitamin B_1, as it does in the case of lactic acid and glucose. It is probable, therefore, that the stimulating action of vitamin B_1 on propionic acid oxidation, noted in Table 4, is partially if not wholly due to the effect of this vitamin on pyruvic acid oxidation. Yet in the case of the oxidation of acetic acid, in which the stimulating action of the vitamin is much more pronounced than in the case of propionic acid, there is no evidence at all of pyruvic acid accumulation in the absence of the vitamin (Table 6).

Experiments were carried out using semicarbazide as a fixative. Pyruvic acid accumulation may be shown to take place in the presence of semicarbazide when the sodium salts of lactic, succinic and propionic acids are oxidized by the vitamin B_1-deficient bacteria. None accumulates, however, when sodium acetate is oxidized under the same conditions. Typical results are shown in Table 7. The absence of any accumulation of a ketonic acid when the bacterial

Table 7

Conditions as in Table 4; *M*/18 semicarbazide hydrochloride present; pH = 7·4; no vitamin B_1 added; 10·5 mg. dry wt. of bacteria.

Substrate present	Pyruvic acid liberated in 1 hr. μl.
0·01 *M* Na lactate	20·0
0·01 *M* Na succinate	20·2
0·01 *M* Na propionate	16·8
0·01 *M* Na acetate	Nil

oxidation of acetic acid takes place in the presence of semicarbazide and in the absence of the vitamin throws doubt on the view that the stimulating action of the vitamin on acetic acid oxidation is connected with the oxidation of pyruvic acid as an intermediate. The following evidence may also be considered.

(1) Examination of the results quoted in Table 3 shows that the ratio of O_2 consumption to acetic acid utilization is approximately 2, even in the absence of added vitamin B_1. If acetic acid were oxidized incompletely in the absence of added vitamin a ratio less than 2 would be expected. Thus, if it were oxidized to succinic acid the ratio would be 0·25, if to malic acid the ratio would be 0·5, and if to pyruvic acid the ratio would be 0·75. Since the experimental ratio is 2·0 the conclusion would be that acetic acid is completely oxidized in the absence of added vitamin B_1, but at a much smaller rate. Since traces of the vitamin must still remain in the bacteria the simplest conclusion accounting for the facts would be that vitamin B_1 is essential for the oxidation of the acetic acid molecule itself. It cannot be concluded from these experiments that pyruvic acid is not an intermediate in the oxidation of acetic acid, for it may be argued that in the

absence of added vitamin the oxidation of acetic acid is so slow that pyruvic acid is formed at a sufficiently feeble rate for it to be fully oxidized with the aid of the traces of vitamin B_1 still present in the bacteria. Admittedly, however, the absence of any accumulation of ketonic acid in the presence of a fixative such as semicarbazide throws doubt on the validity of this argument.

(2) The suggestion may be made that, in the absence of vitamin B_1, traces of intermediates accumulate during the oxidation of acetic acid by propionic acid bacteria and that these intermediates retard the oxidation of the fatty acid. Such intermediates may be succinic acid, *l*-malic acid and pyruvic acid. Experiment shows that the addition of small quantities of these substances to acetic acid has no retarding effect whatsoever on acetic acid oxidation. With succinic and *l*-malic acids an accelerating effect on the oxidation of acetic acid is observed. This phenomenon will be discussed in a later paper. With pyruvic acid no inhibition of acetic acid oxidation occurs, as shown by a typical result given in Table 8, in which pyruvic acid was still present at the end of the respiration

Table 8

Conditions as in Table 1

Substrates present	% acetate utilized in 1 hr.
0·01 *M* Na acetate	10·9
0·01 *M* Na acetate + 5 μg. cocarboxylase	41·5
0·01 *M* Na acetate + 0·0017 *M* pyruvate + 5 μg. cocarboxylase	46·0

period when the acetic acid analyses were made. There was thus ample ketonic acid present throughout the experiment to bring about the alleged retardation of acetic acid oxidation.

(3) If acetic acid oxidation by propionic acid bacteria takes place via the intermediate production of pyruvic acid, it follows from the facts (*a*) that acetic acid is completely oxidized, (*b*) that no pyruvic acid accumulates, that the rate of removal of pyruvic acid by the bacteria should be greater than or at least equal to the rate of utilization of acetic acid under the same experimental conditions. Experiments designed to test this conclusion show that pyruvate is utilized by the bacteria at about the same rate as acetic acid. These results are shown in Table 9. Whilst this result is not inconsistent with the view that

Table 9

Conditions as in Table 4

Substrates added	Vitamin B_1 added μg.	Wt. of bacteria mg.	Substrate removed in 1 hr. μl. gas
0·01 *M* Na acetate	4	13·4	428
0·01 *M* Na pyruvate	4	13·4	504
0·0033 *M* Na acetate	4	10·5	178
0·0033 *M* Na pyruvate	4	10·5	181

pyruvic acid may be formed during acetic acid oxidation, it follows (on this view) that pyruvic acid must be produced from acetic acid at an appreciable rate. It is, therefore, very surprising that none should appear in the absence of vitamin B_1.

(4) If a substrate is oxidized via pyruvic acid, it follows that during the oxidation of the substrate the enzyme dealing with pyruvic acid becomes either partially or wholly saturated with the ketonic acid. This will result in a lowering of the rate of utilization of any pyruvic acid initially added with the substrate, there being in effect less free enzyme available to cope with the added pyruvic

acid. Thus if a mixture of pyruvic acid and a substrate be presented to a bacterial suspension the rate of disappearance of the pyruvic acid will depend on the extent to which the pyruvic enzyme is saturated by the ketonic acid arising from oxidation of the substrate itself.

Experiments, of which typical results are given in Table 10, show that when pyruvic acid (0·0033 *M*) is mixed with lactic acid, succinic acid, fumaric acid or glycerol, vitamin B_1 being present throughout, there is almost complete inhibition of utilization by propionic acid bacteria of the pyruvic acid added initially. These highly inhibitory effects are quite consistent with the fact that these substrates give rise to small but demonstrable quantities of pyruvic acid during their oxidation (Tables 6, 7). They show that pyruvic acid is formed from them at a sufficient rate to saturate the enzyme involved. The admixture of propionic acid with pyruvic acid results in a definite inhibition of the rate of utilization of the latter, as would have been anticipated. The addition, however, of acetic acid to pyruvic acid results in no inhibition within the experimental error of these experiments. This result is opposed to the view that acetic acid is oxidized via pyruvic acid for the following reasons.

(*a*) Acetic acid, as already stated, is oxidized fast enough to produce pyruvic acid at a rate sufficiently high to saturate, partially or wholly, the pyruvic acid enzyme. Propionic acid, which is oxidized at a rate less than that of acetic acid, brings about a definite inhibition of the utilization of added pyruvic acid. The lack of inhibition due to acetic acid indicates that pyruvic acid is being produced from it either extremely feebly or not at all.

(*b*) If acetic acid is oxidized via pyruvic acid, succinic acid is presumably the first product of oxidation of the fatty acid. Succinic acid, however, inhibits the utilization of added pyruvic acid (Table 10). It follows, from the lack of inhibition due to acetic acid, that the rate of formation from it of succinic acid must be small. Considering the relatively rapid rate of oxidation of acetic acid, it seems unlikely, therefore, that it is oxidized to an appreciable extent via succinic and pyruvic acids.

Table 10

Conditions as in Table 1. 4 μg. vitamin B_1 present throughout.

Substrates added	$-Q_{pyruvate}$ (μl. pyruvate utilized per hr. per mg. dry weight bacteria)
0·0033 *M* Na pyruvate	14·5
+0·01 *M* Na *dl*-lactate	0·0
+0·01 *M* Na succinate	2·3
+0·0066 *M* Na succinate	3·9
+0·0033 *M* Na succinate	10·0
+0·01 *M* Na fumarate	0·0
+0·0066 *M* Na fumarate	0·0
+0·0033 *M* Na fumarate	1·4
+0·08 *M* Na malonate	15·1
+0·01 *M* Na formate	17·3
+0·01 *M* Na acetate	14·7
+0·01 *M* Na propionate	11·5
+0·01 *M* Na butyrate	15·4
+0·01 *M* Na α-glycerophosphate	14·1
+0·01 *M* glycerol	0·9

It will be noted from Table 10 that formic acid and butyric acid do not inhibit the utilization of pyruvic acid, nor is the latter affected by the addition of malonic acid or α-glycerophosphoric acid.

The conclusions to be drawn from these experiments are:

(1) Vitamin B_1 is necessary for the oxidation of acetic acid by propionic acid bacteria.

(2) There is as yet insufficient evidence to show that acetic acid is oxidized via pyruvic acid in presence of propionic acid bacteria, the available evidence being against this conclusion.

Oxidation of substrates other than fatty acids by propionic acid bacteria

As may be anticipated, those substrates the oxidations of which are known to proceed through pyruvic acid as an intermediate are oxidized by vitamin B_1-deficient propionic acid bacteria at rates[1] which are increased by the addition of vitamin B_1 to the bacterial suspensions. Typical results are shown in Table 11.

Table 11

Conditions as in Table 1

Substrates added	Q_{O_2} In absence of added vitamin B_1	Q_{O_2} In presence of 4 μg. vitamin B_1
Nil	13·1	16·6
0·01 *M* Na pyruvate	20·5	38·8
0·01 *M* Na *dl*-lactate	35·6	52·8
0·01 *M* Na succinate	29·9	50·2
0·01 *M* Na fumarate	28·3	44·6
0·01 *M* Na *l*-malate	18·9	26·1
0·01 *M* glucose	23·5	34·3
0·01 *M* fructose	28·4	32·2
0·01 *M* Na *l*-glutamate	18·9	25·9
0·01 *M* Na α-ketoglutarate	16·5	18·1
0·01 *M* *dl*-alanine	16·6	20·6
0·01 *M* glycerol	28·8	35·7
0·01 *M* Na α-glycerophosphate	16·9	18·8
0·01 *M* Na hexosediphosphate	21·0	21·0
0·01 *M* Na citrate	15·8	16·8
0·01 *M* Na β-hydroxybutyrate	18·4	22·7

The oxidation of lactic acid by vitamin B_1-deficient propionic acid bacteria is stimulated by the addition of vitamin B_1 to an extent comparable with that occurring with the oxidation of pyruvic acid. Similar large accelerating effects due to the vitamin are to be noted with the oxidation of succinic and fumaric acids. It is, however, remarkable that the rate of oxidation of *l*-malic acid is less than that of fumaric acid and that the effect of vitamin B_1 addition is not nearly so pronounced as with succinic and fumaric acids. This result throws doubt on the generally accepted view that fumaric acid oxidation necessarily proceeds through the intermediate formation of malic acid. The fact that the rate of oxidation of fumaric acid may exceed that of malic acid has been noted before [Quastel & Wheatley, 1931] with *M. lysodeikticus* and *Ps. pyocyanea*.

This result, however, cannot be held as proof that fumaric acid oxidation, in the presence of propionic acid bacteria, proceeds independently of the formation of malic acid, as it is possible that fumarate may act catalytically in this oxidation. Thus, the following reactions may occur.

1. Fumaric acid $\rightleftarrows$ *l*-malic acid.
2. *l*-Malic acid + fumaric acid $\rightleftarrows$ oxaloacetic acid + succinic acid.
3. Succinic acid $\xrightarrow{O}$ fumaric acid.
4. Oxaloacetic acid $\rightarrow$ pyruvic acid + CO_2.

[1] I.e. the rates of oxygen uptake by the bacteria in presence of the substrates.

If reaction 2 is sufficiently rapid, a catalytic action of fumaric acid on malic acid oxidation will occur, its extent being dependent on the initial concentration of fumaric acid present.

A noteworthy fact is that α-ketoglutaric acid undergoes but little oxidation by propionic acid bacteria, its rate being scarcely influenced by the addition of vitamin B_1. Glutamic acid is oxidized at a somewhat faster rate and there is a definite, though feeble, stimulating action of vitamin B_1 on its oxidation. Alanine is but poorly oxidized, and vitamin B_1 has little effect on its rate of oxidation.

Both glucose and fructose are attacked by the vitamin B_1-deficient bacteria, but the addition of the vitamin has a much larger effect on the oxidation of glucose than upon that of fructose under the given experimental conditions.

It is interesting to note (Table 10) that whereas sodium α-glycerophosphate suffers little oxidative change by the bacteria, either in the presence or absence of vitamin B_1, glycerol is strongly attacked, the addition of vitamin B_1 having a marked effect on the rate of O_2 uptake. Sodium hexosediphosphate is oxidized at a rate which is appreciably faster than that of sodium α-glycerophosphate, but the rate is quite unaffected by the addition of vitamin B_1.

Sodium citrate and sodium β-hydroxybutyrate are oxidized but feebly, the addition of vitamin B_1 having little effect on the rates of O_2 uptake in presence of these substances.

Oxidation of ethyl and propyl alcohols by propionic acid bacteria

On testing methyl, ethyl and propyl alcohols as substrates for oxidation by vitamin B_1-deficient propionic acid bacteria, it was found that whereas methyl alcohol is not (or very feebly) attacked, ethyl and propyl alcohols are strongly oxidized. The addition of vitamin B_1 greatly increases the rate of oxidation of ethyl alcohol, but its effect on that of propyl alcohol is very much smaller. Typical results are shown in Table 12. The oxidation of ethyl alcohol leads to the

Table 12

Conditions as in Table 4

Substrate	Q_{O_2} In absence of added vitamin B_1	Q_{O_2} In presence of 4 μg. vitamin B_1
Nil	10·5	12·4
0·033 *M* methyl alcohol	11·5	13·9
0·033 *M* ethyl alcohol	32·2	44·1
0·033 *M* propyl alcohol	61·7	66·2

Table 13

Conditions as in Table 1

Substrate	μl. acetic acid in 1 hr. in absence of added vitamin B_1	μl. acetic acid in presence of 4 μg. vitamin B_1
0·03 *M* ethyl alcohol	201·6	47·0

appearance of a volatile acid, presumably acetic acid. Estimations show that the formation of this acid is greatly reduced when the oxidation of ethyl alcohol by the bacteria takes place in presence of vitamin B_1 (Table 13). Doubtless the stimulant action of vitamin B_1 on ethyl alcohol oxidation is partly due to its powerful effects on the oxidation of acetic acid formed as intermediary.

Oxidation by propionic acid bacteria of substrates nearly related to acetic acid

It was of interest to examine the respiration of propionic acid bacteria in presence of substrates nearly related to acetic acid. Glycollic acid is oxidized at a low rate which is slightly increased by the addition of vitamin B_1. Glycine is oxidized at a higher rate with a small but definite accelerating action due to the addition of vitamin B_1. Neither of these substances is oxidized at a rate comparable with that of acetic acid in presence of the vitamin. Oxalic acid is oxidized either very feebly or not at all. These results are noted in Table 14.

Table 14

Conditions as in Table 4

	Q_{O_2}	
Substrate added	In absence of added vitamin	In presence of 4 μg. vitamin B_1
Nil	10·5	12·4
0·01 *M* glycine	22·4	29·5
0·01 *M* Na glycollate	15·0	19·0
0·01 *M* Na oxalate	11·2	13·7
0·01 *M* Na acetate	23·4	65·3

Vitamin B_1 and the oxidation of α-ketobutyric acid

The addition of sodium α-ketobutyrate to vitamin B_1-deficient bacteria results in a rate of O_2 uptake approximately the same as that which occurs in the presence of sodium pyruvate. The addition of vitamin B_1, however, under the given experimental conditions results in little or no stimulation of the respiratory rate, in contrast to that which occurs with sodium pyruvate (see Table 15). Long &

Table 15

Conditions as in Table 1

	Q_{O_2}	
Substrate added	In absence of added vitamin B_1	In presence of 4 μg. vitamin B_1
Nil	13·1	16·6
0·01 *M* Na pyruvate	20·5	38·8
0·01 *M* Na α-ketobutyrate	19·0	21·7

Peters [1939] found that with washed avitaminous pigeon brain the respiratory rate in presence of sodium α-ketobutyrate is stimulated to a small extent by the addition of vitamin B_1. Under different experimental conditions, which will be described in a later paper, we have found that there is a definite effect of vitamin B_1 on the oxidation of sodium α-ketobutyrate by the propionic acid bacteria. The effect, however, is much smaller than that occurring with pyruvic or acetic acid.

Vitamin B_1 and cocarboxylase

Cocarboxylase is approximately as effective as vitamin B_1 in stimulating the rate of O_2 uptake by vitamin B_1-deficient bacteria in presence of sodium acetate. A characteristic feature of its activity, however, as is shown by typical results given in Table 16, is that its effects are a little less marked than those of vitamin B_1 at small concentrations and a little more marked at high concentrations. The fact

Table 16

Conditions as in Table 4. Substrate: 0·01 *M* Na acetate

	Q_{O_2}			
	0·3 μg.	1 μg.	5 μg.	10 μg.
Vitamin B_1	48·0	50·5	50·5	52·2
Cocarboxylase	37·6	41·8	53·4	56·2

that cocarboxylase activity exceeds that of vitamin B_1 at relatively high concentrations is evidence in favour of the conclusion that cocarboxylase, rather than vitamin B_1, is the actual catalytic agent. Silverman & Werkman [1939, 1, 4] have shown that propionic acid bacteria synthesize cocarboxylase from vitamin B_1. The fact that the activity of cocarboxylase in stimulating the respiration of propionic acid bacteria in presence of sodium acetate is feebler than that of vitamin B_1 at small concentrations may be attributed to a smaller rate of penetration of the pyrophosphoric acid ester into the bacterial cell.

Lipmann [1937] demonstrated the importance of cocarboxylase in the respiration of alkali-washed cells of *Bact. Delbruckii* in presence of sodium pyruvate. Barron & Lyman [1939] have found that cocarboxylase, rather than vitamin B_1, is the effective catalytic agent in the respiration of *Streptococcus haemolyticus* and of *M. gonorrhoeae* in a pyruvate medium.

Effects of components of vitamin B_1 on acetate oxidation by vitamin B_1-deficient propionic acid bacteria

It has been shown by Knight [1937, 1] that a mixture of 4-amino-5-aminomethyl-2-methylpyrimidine and 4-methyl-5-β-hydroxyethylthiazole is almost as effective as vitamin B_1 itself on the growth of staphylococci. Hills [1938] found that a mixture of the two components secures a large increase of O_2 taken up by vitamin B_1-deficient staphylococci in presence of sodium pyruvate. The components separately have no significant effect in raising the O_2 uptake.

A similar effect is found of the mixture of the two components on the oxidation of sodium acetate by vitamin B_1-deficient propionic acid bacteria. Typical results are shown in Table 17.

Table 17

Conditions as in Table 1. Substrate = 0·01 *M* Na acetate

Additions to the medium in the respiration flask	μl. O_2 uptake in 1 hr.
Exp. 1	
Nil	211·9
100 μg. pyrimidine component	215·6
100 μg. thiazole component	226·3
100 μg. pyrimidine component + 100 μg. thiazole component	435·0

Conditions as in Table 4. 0·01 *M* acetate throughout

Exp. 2	Q_{O_2}
Nil	28·8
0·2 μg. pyrimidine component	39·8
0·2 μg. thiazole component	27·5
0·2 μg. pyrimidine component + 0·2γ thiazole component	60·5
0·2 μg. vitamin B_1	62·3

These results make it clear that propionic acid bacteria are able to synthesize vitamin B_1 from a mixture of the two components, even when these are present

in low concentrations. Moreover, the results demonstrate that the components of vitamin B_1 are unable, separately, to exert catalytic effects on the oxidation of acetic acid by propionic acid bacteria. We have, however, found that under certain conditions the pyrimidine component can itself bring about an apparent stimulation of acetic acid oxidation (see, for example, exp. 2, Table 17). This finding is being further investigated. The phenomenon is probably similar to that described by Ochoa & Peters [1938] and explained by Westenbrink & v. Dorp [1940]. Silverman & Werkman [1939, 3] were unable to show a synthesis of vitamin B_1 from a mixture of the pyrimidine and thiazole components by propionic acid bacteria. Their pyrimidine component, however, differed from the one used in this work in having a $—CH_2Br$ group attached to the 5C atom of the pyrimidine ring instead of $—CH_2NH_2$. Conceivably, as Silverman & Werkman point out, linkage may not take place when the former group is attached to the pyrimidine ring.

Summary

1. The presence of vitamin B_1 at low concentrations (e.g. *ca.* $10^{-7}M$) greatly increases the rate of oxidation of acetic acid by vitamin B_1-deficient propionic acid bacteria. The ratio of mol. oxygen consumed to mol. acetic acid utilized by these bacteria, in the presence and in the absence of vitamin B_1, is approximately 2·0, showing complete oxidation of acetic acid.

2. Formic, acetic, propionic and butyric acids are oxidized at about the same rate by vitamin B_1-deficient propionic acid bacteria. The addition of vitamin B_1 has little or no effect on the oxidation of formic and butyric acid, but has a definite stimulating action on that of propionic acid. This effect is much less than that observed with the oxidation of acetic acid.

3. Pyruvic acid accumulates during the oxidation of glucose, lactic acid, glycerol and propionic acid by vitamin B_1-deficient propionic acid bacteria. Its amount greatly diminishes in presence of the vitamin. No pyruvic acid accumulates when acetic acid is oxidized.

4. In presence of semicarbazide a ketonic acid (probably pyruvic acid) accumulates during the oxidation of lactic, succinic and propionic acids. It does not accumulate during the oxidation of acetic acid.

5. The rate of utilization of acetic acid by propionic acid bacteria in presence of vitamin B_1 is approximately equal to that of pyruvic acid.

6. The presence of lactic acid, succinic acid, fumaric acid or glycerol greatly inhibits the utilization of pyruvic acid, when this is added together with any of these substrates, by propionic acid bacteria in presence of vitamin B_1. Propionic acid also produces a definite inhibition of added pyruvic acid utilization, but acetic acid gives no inhibition within limits of experimental error.

7. The presence of vitamin B_1 greatly stimulates the rates of respiration by vitamin B_1-deficient propionic acid bacteria with the following substrates: pyruvic acid, lactic acid, succinic acid, fumaric acid, glucose, glycerol. Definite but much less powerful effects of the vitamin are shown in the oxidations of *l*-malic acid, *l*-glutamic acid, *dl*-alanine, glycollic acid, glycine and fructose. The following substances are oxidized either feebly or not at all by the bacteria, with little or no stimulating action of vitamin B_1: oxalic acid, citric acid, β-hydroxybutyric acid. Oxidation of α-glycerophosphoric acid and hexosediphosphoric acid takes place with little or no effect of added vitamin B_1 on their rates of oxidation. Oxidation of fumaric acid takes place at a greater rate than that of *l*-malic acid.

8. The stimulant action of vitamin B_1 may, in most cases, be attributed partially if not wholly to its effect on the oxidation of pyruvic acid formed as an

intermediate. The effect of the vitamin on acetic acid oxidation cannot at present be ascribed to this cause.

9. α-Ketobutyric acid is oxidized slowly by vitamin B_1-deficient propionic acid bacteria, there being little acceleration on addition of the vitamin.

10. Ethyl alcohol is oxidized by vitamin B_1-deficient propionic acid bacteria, the rate of oxidation in its presence being greatly stimulated by the addition of vitamin B_1. Acetic acid accumulates during the oxidation of the alcohol, its amount being greatly reduced in presence of vitamin B_1. Probably the accelerating action of the vitamin on ethyl alcohol oxidation is due to its powerful effects on the oxidation of acetic acid formed as intermediary. Propyl alcohol is vigorously oxidized by vitamin B_1-deficient bacteria, but there is relatively little accelerating effect due to the addition of vitamin B_1. Methyl alcohol suffers little oxidative change in the presence or absence of vitamin B_1.

11. The evidence is in favour of the conclusion that cocarboxylase is the effective catalytic agent.

12. The pyrimidine and thiazole components of vitamin B_1 are, separately, unable to catalyse the oxidation of acetic acid by propionic acid bacteria. Together they are as effective as vitamin B_1 or cocarboxylase. Thus linkage of the two components will take place in presence of propionic acid bacteria.

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20. THE TRYPTOPHANASE-INDOLE REACTION

4. SOME OBSERVATIONS ON THE PRODUCTION OF TRYPTOPHANASE BY *ESCH. COLI*; IN PARTICULAR THE EFFECT OF THE PRESENCE OF GLUCOSE AND AMINO-ACIDS ON THE FORMATION OF TRYPTOPHANASE

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FILDES [1938], referring to the claims of Happold & Hoyle [1936] that 'the failure to produce indole by cultures of *E. coli* grown in the presence of glucose is due to the non-production of a tryptophanase system', suggests that this is but a partial truth and that the tryptophanase system in the cells consists of two parts, a 'constitutive' part and an 'adaptive' part, of which only the 'adaptive' part is not produced in the presence of glucose. Fildes further states that suspensions of *E. coli* are always capable of catalysing the oxidation of tryptophan to indole even when grown in the absence of tryptophan or in the presence of glucose and tryptophan.

The main points of difference in technique and findings between the two series of investigations are: Fildes obtained his cells from cultures grown on a synthetic medium containing glucose and tryptophan and reported that indole was formed in these cultures and that the suspensions derived therefrom when incubated with tryptophan produced indole. Happold & Hoyle, having sought to explain the absence of indole formation which occurs in glucose bouillon cultures, grew their cells in this medium; indole was not produced in such cultures nor was there enzyme formation, since when non-viable preparations from these cells were placed in contact with tryptophan, indole was not formed, though similar preparations from bouillon cultures were active. The latter technique does not allow neo-enzyme formation to occur during the course of the experiment.

Both series of investigations have been repeated and are experimentally correct. Fildes' results may be due to neo-enzyme formation by the viable cell suspension in the presence of tryptophan as substrate and the absence of glucose. Certainly there are as yet no grounds for postulating a separate 'constitutive' tryptophanase system.

The experimental evidence which supports the above thesis was obtained as follows. Use was made of the previously observed phenomenon that the viability of washed cells of *E. coli* can be destroyed by the addition of a small amount of toluene immediately after the addition of the substrate and that this procedure inactivates only a proportion of the tryptophanase already formed, provided that the relative concentration of the toluene to the cells does not become too high. Treatment of the cells with toluene destroys their ability to cause carbohydrate breakdown.

[1] In part from the Department of Bacteriology, University of Birmingham.

Experimental

The Jebbs strain of *E. coli* (previously supplied by Prof. McIntosh) was used in all experiments. It was inoculated into media from weak suspensions prepared by suspending an agar colony of *E. coli* in 10 ml. sterile saline. The suspensions used for the enzyme work were gathered from 18–24 hr. cultures at 37°. Those taken from solid media were washed off with a mixture of 0·5 % saline and *M*/60 phosphate buffer (*p*H 7·0) and the cells separated from the suspending fluid by a high-speed centrifuge. The cells were washed twice in the saline phosphate mixture and finally suspended in it. Those taken from fluid media were separated from the medium, washed twice with the saline phosphate mixture and also suspended in the same manner. Glucose, where used, was added to the medium as a sterile solution to give final concentrations of 0·5–1·0 %. The details for growth experiments are given separately.

The non-production of the tryptophanase system in glucose bouillon agar cultures of E. coli

Washed suspensions from (*a*) two Roux bottles of bouillon agar and (*b*) two Roux bottles of bouillon agar +1 % glucose were used. Suspensions and substrate were incubated separately at 37° for 15 min. before mixing. The following

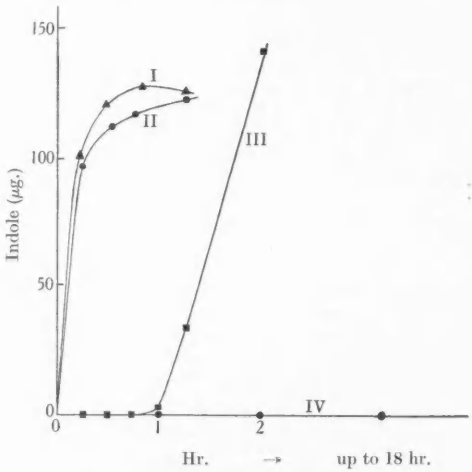

Fig. 1 Indole production from tryptophan by *E. coli*. I, *coli* cells from bouillon agar. II, *coli* cells from bouillon agar toluenated. III, *coli* cells from bouillon agar +1% glucose. IV, *coli* cells from bouillon agar +1% glucose toluenated.

series of flasks was then set up, each flask containing 250 μg. tryptophan in 10 ml. of buffer mixture:

Series 1. Substrate +5 ml. of cell suspension from bouillon agar culture.
Series 2. Substrate +5 ml. of cell suspension from bouillon agar culture +0·2 ml. toluene.
Series 3. Substrate +5 ml. of cell suspension from glucose bouillon agar culture.
Series 4. Substrate +5 ml. of cell suspension from glucose bouillon agar culture +0·2 ml. toluene.

The final turbidity of the suspensions was 2500 million cells per ml. The results are shown in Fig. 1.

The above series of results is characteristic of many similar experiments except that it represents one where there was no appreciable inactivation of the enzyme by the toluene. It will be observed that 87 % conversion of tryptophan into indole occurred as a result of the activity of cells grown in the absence of glucose, that in the glucose series there was practically no enzymic action after 1 hr. but that the reaction was 97 % complete after a further 2 hr. incubation, and finally that no indole was produced in the toluene-treated glucose agar suspensions. The only variations in results from many similar experiments of the above type are: (*a*) that inhibition of indole production in series (3) varies in lasting from 45 min. to 2 hr. after the addition of the substrate (this is probably due to the metabolism of stored carbohydrate); (*b*) that suspensions grown in the absence of glucose and treated with toluene are normally less active than their corresponding viable cells in series (1) by anything up to 60 %, depending on the cell concentration. This is shown in the following results, which are expressed in μg. units:

	30 min.	60 min.	120 min.	180 min.
(1) With 250 μg. tryptophan, after:				
Washed suspension	20	30	50	60
Washed suspension + 0·2 ml. toluene	+	15	17	17
(2) With 1000 μg. tryptophan:				
Washed suspensions	Rapid rise to 119		—	100
Washed suspensions + 0·2 ml. toluene	Rapid rise to 55		—	62

The greatest variation has been obtained with washed cells grown in Fildes' medium + glucose and tryptophan. Frequently, we have obtained washed cells which have not produced more than 1–2 μg. indole from 250 μg. tryptophan in 16 hr.; frequently we have had rapid indole production with the washed cells, but always after a lag period, never have we obtained any indole formation from washed cells which have had toluene added after the addition of the substrate. An example of the second type of result is given in the following table:

Cells grown in 200 ml. Fildes' medium + glucose + tryptophan

	Indole in μg.			
	20 min.	30 min.	40 min.	60 min.
Washed cells + 250 μg. tryptophan	Nil	10	75	125
Washed cells + 250 μg. tryptophan + 0·2 ml. toluene	Nil	Nil	Nil	Nil

It will be observed that the results with the washed cells obviously fall on an autocatalytic curve as do those with the glucose bouillon agar suspensions and that adaptation to the substrate is taking place. The metabolism of stored carbohydrate is also progressing in the suspensions which are free from toluene. Cells grown in Fildes' medium alone (no tryptophan) are devoid of tryptophanase activity.

It has again been confirmed that fermenting glucose does not stop the tryptophan-tryptophanase indole reaction when the tryptophanase is already present before the glucose is added, and further that the tryptophan content of a medium does not decrease when *E. coli* is grown in it in the presence of glucose, at least whilst other amino-acids are present.

The former point is demonstrated by the following experiment. Twice-washed suspensions of organisms grown on bouillon agar were incubated in four 250 ml. flasks each containing 1 % glucose and 2 mg. of tryptophan per 50 ml. Two of the flasks contained 1 ml. of toluene in each flask. Glucose determinations showed that glucose was disappearing from the non-toluenated flasks during the course of the experiment. The agreement using this technique is not so good as

when separate flasks are used for each determination, but the results are adequate for the purpose required:

Period of incubation	Indole produced per 10 ml. in μg.			
	Without toluene		With toluene	
	Flask (1)	Flask (2)	Flask (3)	Flask (4)
30 min.	51	41	21	22
1½ hr.	83	69	37	44
3½ hr.	100	83	41	41
5¼ hr.	101	95	50	44

The non-disappearance of tryptophan from the culture medium when *E. coli* is grown in the presence of glucose, tryptophan and other amino-acids, i.e. when tryptophanase is not formed, was demonstrated by means of an eight-amino-acid medium [Evans *et al.* 1939] as follows:

Four flasks of the above eight-amino-acid culture medium were set up: (1) Basal medium uninoculated. (2) Basal medium inoculated with *E. coli*. (3) Basal medium + 0·5 % glucose uninoculated. (4) Basal medium + 0·5 % glucose inoculated with *E. coli*.

After 23 hr. incubation at 37°, 10-ml. samples from the flasks showed no change in reaction except that from flask (4) which was acid (*p*H 5). Tryptophan estimations on samples (by the method of Lugg [1937]) gave the following results:

	Tryptophan as g. per 100 ml.			
	Flask (1)	Flask (2)	Flask (3)	Flask (4)
After 23 hr.	0·0112	0·0019	0·0117	0·0111

Although the inoculated flasks (2) and (4) had both yielded a good growth of *E. coli* tryptophan had only disappeared from flask (2): this was the only flask which gave any reaction for indole and in this case it was a very strong reaction.

From the above experimental evidence it seems certain that the presence of tryptophan is essential for the production of tryptophanase, that where there is no indole formation in cultures containing glucose and tryptophan there is no tryptophanase production. Further, since resting bacteria do not apparently rest (but are capable of giving rise to neo-enzyme formation) the washed viable cell technique is unsafe as a method of analysis for the presence or absence of an enzyme at a given point in time.

Specific inhibition of indole production by E. coli *in the presence of phenylalanine or tyrosine and glucose in simple media*

There remains the fact, however, that under certain conditions, e.g. when grown in Fildes' medium + tryptophan, the inhibitory effect of glucose on the production of indole by cultures of *E. coli* is not complete, up to one-fifth of the indole produced in the absence of glucose being formed. This appears to be due to the peculiarities of the constitution of the medium.

It has been found that complete inhibition of indole formation in the presence of glucose and tryptophan in Fildes' medium can be obtained by adding 10 ml. of a 15 % gelatin solution per 50 ml. of Fildes' medium. Further, it was found that if the gelatin were replaced by a mixture of amino-acids representing those found in gelatin by analysis the same result was obtained. Each of the amino-acids found in gelatin hydrolysates was now added separately to Fildes' medium + glucose + tryptophan to a concentration of 0·01 %. Of the amino-acids used—

glycine, alanine, leucine, aspartic acid, glutamic acid, phenylalanine, cystine, lysine, arginine, histidine, proline and hydroxyproline—only phenylalanine and tyrosine (this was used later) completely inhibited indole production. At the concentration employed (0·01 %) the other amino-acids had no influence on the amount of indole formed. Further, it has been found that in the case of mono Na glutamate, leucine, valine, glycine, alanine, aspartic acid and histidine concentrations of 0·05, 0·10, 0·15 and 0·20 % have no effect on the production of indole in the presence of glucose. It has been demonstrated that phenylalanine and tyrosine do not affect the amount of indole produced from tryptophan in the absence of glucose. Numerous growth experiments have been carried out to determine the concentrations of phenylalanine and tyrosine necessary to complete the inhibition of indole production by glucose in Fildes' medium. The following relationships hold:

0·001 % tryptophan = 0·001–0·002 % tyrosine.
= 0·003–0·004 % phenylalanine.

0·002 % tryptophan = 0·003–0·004 % tyrosine.
= 0·005–0·006 % phenylalanine.

0·003 % tryptophan = 0·004–0·005 % tyrosine.
= 0·007 % phenylalanine.

There is evidence here of a possible stoichiometric relationship between the tryptophan and the tyrosine and a less exact relationship between the phenylalanine and the tryptophan (the phenylalanine is a synthetic amino-acid).

The action of glucose + phenylalanine or tyrosine on preformed tryptophanase has also been studied.

Washed suspensions of *E. coli* grown on bouillon agar were prepared and added to flasks containing buffer solution *p*H 7·6 and 0·01 % phenylalanine or tyrosine sterilized by autoclaving as basal substrate. The following experimental flasks were set up: 30 ml. buffer solution and 30 ml. of cell suspension were placed in all the flasks:

(1) 1·5 ml. 1/1000 sterile tryptophan.
(2) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine.
(3) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine + 1 ml. toluene.
(4) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine + 1 ml. 50 % sterile glucose.
(5) 1·5 ml. 1/1000 sterile tryptophan + 0·05 % phenylalanine + 1 ml. 50 % sterile glucose + 1 ml. toluene.

The tryptophan was added last to all the flasks, which were then incubated at 37°. 10-ml. samples were taken from each flask, ½, 1 and 3 hr. after the start of incubation. The results were as follows:

	Indole ½ hr.	Reaction		
		1 hr.	3 hr.	
Flask no.	μg.	μg.	μg.	Reaction
(1)	76	119	124	No change
(2)	76	120	125	,,
(3)	17	40	39	,,
(4)	76	110	108	Slightly acid
(5)	17	45	44	No change

Similar results obtained with tyrosine show that in the presence of phenylalanine and tyrosine preformed tryptophanase produced almost theoretical yields of indole from tryptophan and also that in the presence of fermenting glucose this yield is not reduced. It should be added that experiments performed for us by Mr H. Anderson show that cells of *E. coli* do not deaminate phenylalanine.

It is clear to us from these and other findings that the effect of the phenylalanine and tyrosine is not exerted through a competitive action upon the enzyme system when formed, but that it must be exerted during the stage of cell division when metabolism is in its most active phase. At the same time the specific effect of these two amino-acids, which show a structural relationship to tryptophan, raises points of interest in view of the findings of Baker & Happold [1940], that the integrity of the alanine group attached to the heterocyclic indole ring is essential to indole formation by suspensions of *E. coli*. It will also be noted that phenylalanine and tyrosine are two of the small group of amino-acids which are regarded as ketogenic in the mammalian metabolism of amino-acids.

It should be unnecessary to add that the term 'tryptophanase' is used, not to imply a single enzyme but to describe a coupled oxidative deamination and decarboxylation in an enzymic system which is specific for the tryptophan-indole reaction.

SUMMARY

It is further confirmed that the presence of tryptophan in a medium is essential for the production of the tryptophanase system by cultures of *E. coli*, and that the enzyme system does not exist as such in cells which have been grown in glucose bouillon agar or glucose tryptic digest agar, but that such cells when freed from glucose by washing will re-develop the enzyme system when left in contact with tryptophan.

Cells grown in complex amino-acid media plus glucose and tryptophan will produce some indole if phenylalanine and tyrosine are absent from such media. There is evidence of a stoichiometric relationship between the amount of tyrosine (?phenylalanine) which inhibits indole production and the tryptophan in media containing an increasing amount of tryptophan. This action is restricted in its effect to the growing cell. Phenylalanine and tyrosine alone or with glucose do not affect the tryptophanase activity of washed cells.

Acknowledgement is made to the Medical Research Council for a grant to one of us (F. C. H.).

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21. HISTIDINE METABOLISM IN TOXAEMIA OF PREGNANCY. ISOLATION OF HISTAMINE FROM THE URINE OF PATIENTS WITH TOXAEMIA OF PREGNANCY[1]

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(*Received 16 December 1940*)

In the course of various investigations it has been demonstrated that histidine is a normal constituent of the urine of pregnant women, that it appears in the urine at a very early stage of pregnancy and continues to be excreted during the whole of gestation [Kapeller-Adler, 1933; 1934; 1936]. The rate of histidine excretion seems to vary with the individual and to be dependent on the kind of food taken [Kapeller-Adler & Schiller, 1935]. No histidine is to be detected in the urine of pregnant animals [Kapeller-Adler & Herrmann, 1934]. Further studies revealed the existence of a relationship between the excretion of histidine and that of gonadotropic hormones in human pregnancy [Kapeller-Adler *et al.* 1935; 1936; 1937]. The increase in the gonadotropic hormones during human gestation was assumed to be the cause of the non-destruction of histidine so that this amino-acid passes the liver unaffected and, not being deaminated by the kidneys, is excreted in the urine. Whereas histidinuria is characteristic of normal human pregnancy a more or less distinct alteration in histidine metabolism occurs in toxic pregnancy. The clinical importance of this alteration is considered in a paper at present in the Press [Kapeller-Adler, 1940]. Briefly the results obtained in the study of 58 cases of toxaemia of pregnancy of various types indicate that histidinuria is not appreciably affected in cases of mild toxic pregnancy, but is considerably diminished in patients with serious symptoms of pre-eclamptic toxaemia; in these little or no histidine is to be found in the urine. So constant is this that a marked diminution in histidine excretion can be used as a diagnostic sign of severe toxaemia of pregnancy.

It seemed possible that in severe toxaemia the histidine instead of being excreted as such was being transformed into some other substance, probably one of pharmacological activity. In this connexion decarboxylation of histidine to histamine had obviously to be considered, and several attempts have been made to detect histamine in the urine from toxaemic patients as well as from cases of normal pregnancy. In this paper these attempts are described.

Experimental

The method used depended upon precipitation of the histamine with $Zn(OH)_2$ [compare Ackermann, 1939], decomposition of the precipitate with H_2S, extraction of the resulting alkaline solution with amyl alcohol, re-extraction of the histamine from the amyl alcohol with $N/10$ H_2SO_4 or $N/10$ HCl, evapora-

[1] The substance of this paper was reported at a Meeting of the Biochemical Society in Edinburgh on 8 June 1940.

tion of this solution *in vacuo* and preparation of the histamine picrate or flavianate.

Exp. 1. 23 l. of urine of four patients with signs of severe toxaemia and no histidinuria were worked up. The urines were collected over chloroform and kept in the ice chest. Every 1000 ml. of the mixed urine were treated with 30 ml. of a saturated solution of freshly precipitated $Zn(OH)_2$ in ammonia (sp. gr. 0·880). After standing overnight the precipitate was filtered off, thoroughly washed with water and dried in air. The dry white powder was treated with dilute H_2SO_4 and freed from zinc by H_2S. The ZnS was filtered off and carefully washed with water. The filtrate was freed from H_2S by air-suction, made strongly alkaline with NaOH and the NH_3 was removed by distillation *in vacuo* at 40°. This solution, completely free from NH_3 and having *p*H 9·5, was concentrated *in vacuo* to about 100 ml. and shaken 6 times with pure amyl alcohol, 50 ml. being used for each extraction. The combined amyl alcohol extracts were shaken 5 times with $N/10$ H_2SO_4 (in later experiments HCl) using 20 ml. each time. To remove traces of amyl alcohol the sulphuric acid solution was vigorously shaken with 25 ml. of chloroform. The resulting liquid was concentrated to a small volume under reduced pressure and then tested for the presence of histamine as follows:

(*a*) With a small amount of this solution Pauly's diazo reaction [1904] was carried out and a strong positive result was obtained. This very sensitive test, although not specific for histamine, being given also by other iminazole derivatives, seemed however in this case to indicate the presence of histamine because other iminazole derivatives are insoluble in amyl alcohol.

(*b*) Another part of the solution obtained was subjected to the reaction of Zimmermann [1929]. This test, which is specific for histamine, also gave a strong positive result.

(*c*) To the main part of the liquid above described an excess of a concentrated solution of flavianic acid was added. After some time a pale yellow precipitate was formed. The M.P. was 256°. This precipitate was purified by repeated crystallizations from hot water. Ultimately, a fraction of a milligram of a yellow crystalline substance, insoluble in water, was obtained and this had M.P. 260°, not depressed on admixture with an authentic specimen of histamine diflavianate. Under the microscope the material appeared as bright yellow characteristic prisms showing great similarity with crystals of histamine diflavianate. On account of the strong positive result of the Zimmermann reaction in the fluid investigated, and of the fact that the isolated flavianate showed many physical properties characteristic of histamine diflavianate it was assumed that the base present might indeed be histamine. It was of course necessary to obtain further evidence for that assumption.

Exp. 2. 34 l. of urine of several patients with signs of severe toxaemia of pregnancy and no histidinuria were worked up in the manner above described. The flavianate isolated had after repeated recrystallization M.P. 260°, not depressed on admixture with histamine diflavianate. Unfortunately, the yield of the material obtained was again very small, amounting only to a fraction of a milligram. Since the identification of the compound, so far carried out, was not at all satisfactory a further attempt was undertaken to procure a greater yield.

Exp. 3. This time it was decided to start from the urine of only two patients with signs of very severe toxaemia of pregnancy and no histidinuria at all in the hope that under these conditions histaminuria might be so considerable as to allow of histamine being isolated in larger amounts. 52 l. of urine from the two patients were treated in the way described, the only difference being that the amyl alcohol extracts were shaken with $N/10$ HCl instead of $N/10$ H_2SO_4. The

liquid thus obtained was evaporated to dryness *in vacuo* after the traces of amyl alcohol had been removed by chloroform. A hydrochloride was obtained which showed strong positive Pauly and Zimmermann reactions. After recrystallization from hot alcohol prisms were obtained which, under the microscope, seemed very similar to crystals of histamine dihydrochloride. This hydrochloride was converted into a picrate which after several recrystallizations from hot water melted at 238–242° with decomposition and brown-black colouring. It showed no depression of M.P. when mixed with histamine dipicrate. This experiment yielded 2·7 mg. of the picrate which, like histamine dipicrate, was soluble in hot water and insoluble in ether. Under the microscope the crystals of the isolated picrate, consisting of yellow plates, closely resembled those of histamine dipicrate. It may be pointed out that histamine picrate as well as histamine flavianate give directly a strong positive diazo reaction without having been previously freed from picric or flavianic acid. Unfortunately, the amount of the picrate obtained was not large enough for a micro-analysis but X-ray powder photographs of it were kindly taken by Dr C. A. Beevers, Chemistry Department, Kings Buildings, Edinburgh.

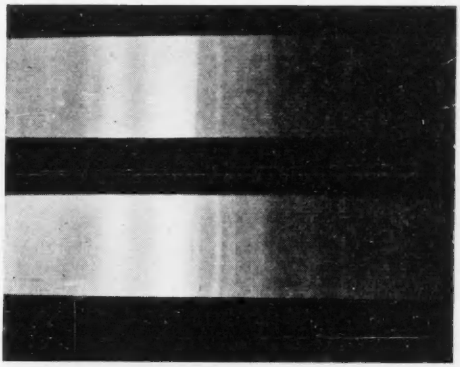

Fig. 1. Photographs of substance I (histamine dipicrate) and substance II (picrate isolated from toxic pregnancy urine).

Dr Beevers makes the following observation on these photographs:

(1) General features identical but II has some extra lines (continuous parts due to glass).

(2) I and II have at least nearly the same crystal structure. What differences there are may be due to (*a*) impurities in II crystallizing in quite a different way; (*b*) imperfect crystallization of I resulting in certain lines becoming blurred. This is supported by the fact that photograph I is generally more blurred than II.

(3) Lines are very numerous, showing a large unit cell (probably large molecules).

(4) Photographs were taken with Cu *K* X-rays (Ni filter to remove K_β), $2\frac{3}{4}$ hr. exposure, 25 mA., 60 kW.).

Exp. 4. Whereas in the previous experiments urines of women suffering from pre-eclamptic toxaemia were used for the isolation of histamine this time the examination of the urine of a woman with hyperemesis gravidarum for the presence of histamine was undertaken.

The urinary output of 6 successive days (3000 ml.) of a woman in the 10th–12th weeks of pregnancy with distinct signs of hyperemesis gravidarum and with almost no histidinuria was the starting material. The urine was daily precipitated with $Zn(OH)_2$ and the precipitate worked up in the manner described above, with only one slight modification. After the decomposition of the zinc salt by H_2S a few ml. of ammonia solution were added to the liquid containing dilute H_2SO_4 in order to ensure complete precipitation of ZnS. The precipitate was allowed to settle overnight and was then worked up according to exp. 3. The hydrochloride obtained showed strong positive diazo and Zimmermann reactions. In presence of a little dilute H_2SO_4 it was converted into the flavianate by means of a concentrated solution of flavianic acid. The bright yellow flavianate showed a strong positive diazo reaction and had M.P. 258°. On several recrystallizations a pure substance was obtained with constant M.P. 260°, not depressed on admixture with an authentic specimen of histamine diflavianate. The yield of the purified material was 10·9 mg. (Found (Weiler): C, 39·70; H, 3·6; N, 13·4 %; required for $C_5H_9N_3(C_{10}H_6N_2SO_8)_2$: C, 40·58; H, 2·86; N, 13·26 %.)

Exp. 5. 39 l. of urine from four normal pregnant women with histidinuria were worked up as in exp. 4. The hydrochloric acid solution finally obtained, although showing a positive diazo reaction, did not yield any histamine diflavianate with flavianic acid. Obviously only traces of histamine were present.

It may be mentioned that Ackermann [1939] has isolated 0·9 mg. histamine dipicrate from 1000 l. of normal urine. Because of the very small amount of the compound he could not identify it accurately.

Discussion

The experiments recorded above indicate that whereas normal pregnancy is characterized by histidinuria, in severe toxaemia of pregnancy the excretion of histamine in the urine seems to replace that of histidine. It should be stressed that the amount of histamine isolated certainly represents only a small part of the amount which may really be present in toxaemic pregnancy urine, since losses in the preparation cannot be avoided and, apart from that, not all the histamine can be recovered.

There is no doubt that the histamine appearing in toxaemia of pregnancy can only be derived from histidine which is normally present in large amounts throughout gestation. Histamine is presumably formed from histidine by decarboxylation. Werle [1936; 1937; 1938] has found that animal tissues, especially the kidneys, contain an enzyme which can alter histidine to histamine by decarboxylation. This enzyme which he called histidine decarboxylase appears to be more effective in absence than in presence of oxygen. But there is still another enzyme in the kidneys with an antagonistic action tending to the destruction of histamine. This enzyme, histaminase, first described and isolated by Best [1929; 1930; 1932; 1935] is effective only in the presence of oxygen.

Although no evidence has been advanced as yet as to the occurrence of histidine decarboxylase in the human body, the above-reported isolation of histamine from toxaemic pregnancy urine seems to suggest the presence of such an enzyme in man. For the conversion of histidine which is excreted in rather large amounts throughout normal pregnancy into histamine appearing in the urine of women with different manifestations of severe toxaemia of pregnancy can only be explained if the presence of a histidine decarboxylase in the human body is assumed.

Accordingly two factors seem to be responsible for the velocity of formation of histamine in histidine metabolism, namely, the activity of the histidine decarboxylase and that of histaminase. In a recent paper Werle [1940] reports that the activities of both enzymes are not only dependent on the presence or absence of oxygen but far more on the presence of various inactivators which regulate their action.

Thus the occurrence of histamine in the metabolism of pregnant women will finally be determined by the balance between histidine decarboxylase and histaminase.

The significance of the isolation of histamine from toxaemic pregnancy urine for the explanation of the pathogenesis and of the course of toxaemia of pregnancy will be discussed in another place.

SUMMARY

1. A chemical method has been described for isolation of histamine from the urine.

2. Four attempts have been made to isolate histamine from the urine of women with various manifestations of toxaemia of pregnancy. Whereas in the first two experiments only fractions of a milligram of histamine diflavianate have been isolated, exp. 3 yielded 2·7 mg. of histamine dipicrate from 32 l. of urine of two women with very severe toxaemia, identified by melting point, mixed melting point, the Zimmermann reaction and an X-ray photograph; in exp. 4, 10·9 mg. histamine diflavianate have been isolated from 3000 ml. of urine of a woman with severe hyperemesis gravidarum and identified by a micro-analysis.

3. From 39 l. of normal pregnancy urine with normal histidine excretion the same method yielded no detectable quantity of histamine.

4. The origin of histamine and the factors responsible for its occurrence in toxic pregnancy are discussed.

I wish to express my gratitude to Prof. F. A. E. Crew and to Dr C. P. Stewart for kind hospitality. To Prof. R. W. Johnstone I am deeply indebted for permission to use the clinical material from his Ward in the Simpson Memorial Maternity Hospital, Edinburgh. My thanks are also due to Prof. G. F. Marrian for his helpful criticism and advice.

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Note added 20 February 1941. Further identification of histamine isolated from the urine of patients with toxaemia of pregnancy.

Since the above paper was submitted more histamine has been isolated from the urine of two toxaemic patients. 7700 ml. of urine of a patient with hyperemesis gravidarum yielded 34·6 mg. histamine diflavianate and 62·5 mg. of the same salt were prepared from 8500 ml. of urine of a woman suffering from serious pre-eclamptic toxaemia. In both cases the histamine diflavianate first obtained was purified and was then freed from flavianic acid by dilute hydrochloric acid and butyl alcohol, thus giving a pure histamine hydrochloride. This salt was converted again into the diflavianate which had M.P. 262–263°, not depressed on admixture with an authentic specimen of histamine diflavianate. (Found (Weiler): C, 39·71; H, 3·22; N, 12·96 %; required for $C_5H_9N_3(C_{10}H_6N_2SO_8)_2$: C, 40·58; H, 2·86; N, 13·26 %.)

Prof. A. J. Clark, Department of Pharmacology of the University, Edinburgh, kindly carried out biological tests with the preparations from both of these patients. In the case of the patient with hyperemesis the pure hydrochloride was used, whilst in the second case the hydrochloride contained traces of the flavianate. In tests on the guinea-pig's ileum, the guinea-pig's uterus and the rat's uterus the preparations from urine were identical in pharmacological action with similar amounts of authentic histamine.